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C.-E. A. WINSLOW

It is characteristic of Science and Progress that they continually open new fields to our vision.—PASTEUR.

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FURTHER STUDIES ON THE GROWTH CYCLE OF AZOTOBACTER[1]

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Ontario Agricultural College, Guelph, Canada

Received for publication February 6, 1920

At the 1912–1913 meetings of the Society of American Bacteriologists the writer read a paper entitled "A Morphological and Cultural Study of Some Azotobacter," using a series of lantern slides of photomicrographs for illustration. The paper was subsequently published in the Centralblatt für Bakteriologie, 1913, and in the "Transactions of the Royal Society of Canada" of the same year.

Among the observations made in this paper was one regarding the formation in azotobacter cells of two types of granules, one type being stainable, and the other non-stainable, with various bacterial stains. The type that was stainable appeared to represent reproductive bodies or gonidia which on disintegration of the mother cell were liberated, after which they grew into normal azotobacter cells and reproduced by fission for a time until later the cells became granular and again disintegrated with the liberation of reproductive granules. The formation of these reproductive granules with their liberation, on the disintegration of the mother cell, appeared to the writer to constitute a new type of multiplication for azotobacter distinct from the usual method of fission characteristic of bacteria in general. As no reference to such a method of multiplication could be found in the literature, the observations were repeated a considerable number of times to verify the above conclusions.

In the Centralblatt for 1914 the writer published another short article entitled "Further Studies with Some Azotobacter."

[1] Presented before the Society of American Bacteriologists, December 29, 1919.

This dealt with the viability, thermal death point and formation of involution forms in four varieties of azotobacter under observation.

The studies with these strains of azotobacter were continued for a short time longer, attention being directed more particularly to the disintegration of mother cells with the liberation of the gonidia-like granules and their subsequent development, and numerous photomicrographs were taken of these phenomena. Unfortunately, these studies were interrupted in the latter part of 1914 and were not resumed until the summer of 1919.

In the meantime Löhnis and Smith published in the Journal of Agricultural Research, July, 1916, a remarkable paper entitled "Life Cycles of the Bacteria." In this article the authors state that the life cycle of many, if not all, species of bacteria is much more complex than has been generally considered to be the case.

The theory which they advance is that

> All bacteria studied live in an organized and in an amorphous stage. The latter has been called the "symplastic stage," because at this time the living matter previously enclosed in the separate cells undergoes a thorough mixing either by a complete disintegration of the cell wall, as well as cell content, or by a melting together of the content of many cells which leave their empty cell walls behind them. In the first case a stainable, in the latter case an unstainable symplasm is produced.
>
> According to the different formation and quality of the symplasm the development of new individual cells from this stage follows various lines. In all cases at first "regenerative units" become visible. These increase in size, turning into regenerative bodies, which later, either by germinating or stretching, become cells of normal shape. In some cases the regenerative bodies also return temporarily into the symplastic stage.
>
> Beside the formation of the symplasm another mode of interaction between the plasmatic substances in bacterial cells has been observed, consisting of the direct union of two or more individual cells. This conjunction seems to be of no less general occurrence than the process first mentioned.

Whilst Löhnis and Smith base their conclusions on many observations which they made on cultures of various species of bacteria including such diverse forms as *B. subtilis* (*Pseud.*)

fluorescens, *Bact. pneumoniae*, *Sar. flava*, *Strep. lactis*, *Lactobacillus bulgaricus* and others, they use the azotobacter life cycle more than any other to demonstrate the truth of their conclusions. They give a diagrammatic representation of the life cycle of azotobacter with drawings of the many types of cell structures observed and suggest how these forms may be related to one another. A number of photomicrographs are also presented to the same end in the article.

Though the writer has not so far observed the complexity of the life cycle in any other species of bacteria than the azotobacter, he has observed it in this species, to a greater or less extent, as pointed out in the articles previously referred to, published in 1913 and 1914. He finds, however, that while he agrees with some, he cannot agree with all the conclusions arrived at by Löhnis and Smith with regard to azotobacter.

SPORE FORMATION BY AZOTOBACTER?

In the first place Löhnis and Smith refer to azotobacter as a heat-resistant endospore-forming bacillus, and point out that another investigator, Mulvania, reports the presence of heat-resisting spores in azotobacter. Further, they state that while other investigators failed to report the finding of resistant spores in azotobacter, "They undoubtedly would have found them by a more thorough search." Löhnis and Smith observed two types of spore-forming rods in the complex cycle of the azotobacter, one a small rod and the other a large rod. They also observed non-spore-forming rods both small and large, but neither of these ever developed directly into spore-formers, although the small spore-forming rod sometimes developed into the large spore-forming rod.

They imply that the faculty of heat-resistant endospore production is not constant in the azotobacter as only about 50 per cent of their cultures possessed it and most of these had developed it only after being kept in the laboratory for a number of years.

When the writer was isolating from the soil the varieties of azotobacter which he studied, he frequently found spore-forming rods, both large and small, cropping up in his cultures

and subcultures. But after a prolonged series of alternate replatings and flask cultivations, these spore-forming rods appeared to be eliminated. It was a comparatively simple matter to isolate the spore-forming rods from the azotobacter, but very difficult to isolate the azotobacter from the spore-forming rods. The spore-forming rods on subsequent cultivation proved to be other species than azotobacter.

As stated in the article "Further Studies of Some Azotobacter" previously mentioned, the writer tested the thermal death point of four varieties of this organism from a series of cultures ranging in age from sixteen days to two years and two months, and in every case the cultures heated to 65°C. or over, failed to give any subsequent growth.

In July, 1919, the stock cultures of azotobacter on Ashby's agar that had remained untouched since 1914, or earlier, were again tested for thermal death point in the recognized manner, except that 10 cc. of Ashby's solution was used instead of water or bouillon in the test tubes that were heated. In every case a generous loopful of the culture was transferred to the test tubes, which, on being stirred in, was not thoroughly broken up, as a considerable number of macroscopic particles of culture remained unbroken, thus favoring resistance to heat on the part of the organisms constituting these particles. Again all cultures heated to 65°C. and over failed to give any subsequent azotobacter growth, while all controls gave good azotobacter development. Thus it was concluded that these four varieties of azotobacter even in cultures that had been kept for four years at room temperature, did not produce heat-resistant endospores. However, on plating out on beef peptone agar from these stock cultures, some of them produced colonies of encapsulated spore-forming rods both large and small. These on isolation and subculture did not develop azotobacter characteristics. On Ashby's agar or in Ashby's solution some would not grow at all and others only to a very limited extent, while on beef media they made good development. It was therefore concluded that the resistant spore-formers, which were found present only in very limited numbers and not in all the cultures tested, were contaminations.

SYMPLASTIC STAGE

With regard to the theory of the symplastic stage in the life cycle of azotobacter described by Löhnis and Smith, our observations of the four varieties of azotobacter studied lead us to conclude that there are good grounds for accepting the theory. Such clusters of cells referred to by Löhnis and Smith as "symplasm" have been observed by us in various stages from clusters in which the individual cells were well defined to clusters in which it was difficult to distinguish individual cells but in which minute granules were present in considerable numbers, evidently corresponding to the reproductive granules of Löhnis and Smith. These granules were readily responsive to some stains, particularly Heidenhain's iron haematoxylin, which stains them black, and Neisser's blue, which stains them dark blue, and makes them quite distinct from the surrounding substance. In other masses of "symplasm" these granules were larger in size and were assuming the appearance of individual cells, and in still others they had become small individual azotobacter cells, multiplying by fission.

Previous to the publication of Löhnis' and Smith's symplastic theory, we had considered these clusters to be mechanical agglomerations of cells, their association being not vital but accidental, and the formation of the reproductive granules with their subsequent liberation being identical with that which we described in 1914 as occurring in individual azotobacter cells.

However, from the observations that we have made this year in connection with our cultures of azotobacter, we have come to the conclusion that there are good grounds for accepting Löhnis' and Smith's theory regarding the fusion or mixing together of the protoplasm of those cells which constitute these symplastic clusters. We have observed these symplastic masses in stained preparations from cultures ranging in age from a few days to several months, on beef peptone agar, Ashby's agar and in Ashby's solution, and, notwithstanding the fact that at first we were antagonistic to the theory, we were finally led to accept it by our repeated observations.

CONJUNCTION OF AZOTOBACTER CELLS?

With regard to the claim put forward by Löhnis and Smith that "In addition to the formation of symplasm another mode of interaction between the plasmatic substances in bacterial cells occurs, consisting of the direct union of two or more cells, which conjunction seems to be of no less general occurrence than the process first mentioned," we cannot altogether agree.

In support of their contention Löhnis and Smith present a number of photomicrographs of stained azotobacter preparations. In studying these photomicrographs and comparing them with our own preparations we come to the conclusion that what is here referred to as conjunction of two individual cells is rather the incomplete fission of individual cells in process of division. This process has been observed by us repeatedly, not only in stained preparations, but also in living cultures on agar hanging block, and in hanging drop cultures in moist chambers inoculated from cultures varying in age from one day to several months. In none of these did we observe two cells unite in conjunction, but many times have we observed fission take place in which the cells presented an appearance similar to that shown as "conjunction" in the pictures of Löhnis and Smith.

INVOLUTION FORMS

Löhnis and Smith object to the use of the generally accepted term "involution forms" as applied to those aberrant or abnormal, usually enlarged swollen forms of bacteria that are liable to occur in cultures of most species of bacteria when grown under varying conditions. They contend that "The development of the bacteria is characterized not by the *irregular* occurrence of more or less *abnormal* forms but by the *regular* occurrence of many different forms and stages of growth connected with each other by *constant relations*."

The term "involution forms," the writer understands, is applied to bacterial cells that have assumed swollen, bladder-like, elongated or irregular forms as a result of variations in

temperature or changes in the culture media in which they are growing, and that cells of this type are weakened or degenerated and sometimes devitalized. This being the case, the writer finds that with azotobacter, in addition to there being a "regular occurrence of many different forms and stages of growth connected with each other by constant relations," there is also an irregular occurrence of more or less abnormal forms which merit the term "involution forms." These forms, in great variety of size and shape, are fairly common in old cultures (one to two months) of azotobacter grown in Ashby's solution or on Ashby's agar at room temperature, but when cultures are incubated at 37°C., they become numerous in a few days. Numerous Ashby's agar hanging block cultures in moist chambers were made by the writer in which various involution forms were included in the inoculum. The development of these cultures was observed under the oil immersion lens and in only a very small percentage of cases did the involution forms show any tendency to develop or reproduce. In most cases the involution forms remained dormant either until they were overgrown by colonies of azotobacter developing near them from normal cells included in the inoculum, or until the cultures gradually dried out. Some of these moist chamber cultures were held under observation for fourteen days, sterile water being added to the chamber from time to time to prevent, as long as possible, the drying out of the culture.

In those cases where the abnormal forms reproduced, the process of reproduction at first more closely resembled budding than ordinary simple fission. Irregular rounded projections would develop, apparently from any part of the cell, and in course of a few hours abstriction of these projections would take place, the abstricted portions then proceeding to develop and reproduce by fission.

SUMMARY AND CONCLUSIONS

1. The four varieties of Azotobacter studied by us, which were isolated from garden soil at Guelph, Ontario, in 1910, and were reported on in 1913 and 1914, have a complex life cycle.

2. Individual cells following a period of reproduction by fission, may develop reproductive granules or gonidia, within their cell plasma, which, on disintegration of the mother cell are dispersed, increase in size, become typical azotobacter short rods, ovals or spheres, and reproduce by fission. The young cells are motile.

3. The reproductive granules vary in size, some being very minute. Attempts to pass them through a Berkfeld filter were not successful. They are positive to Neisser's blue and to Heidenhain's iron haemotoxylin stains.

4. Another type of granule, not reproductive and not stainable, possibly composed of reserve food substance, is found associated with the reproductive granules in the mother cell.

5. A "symplastic stage" as described by Löhnis and Smith, has been observed in cultures varying in age from a few days to several weeks. In this symplastic stage aggregations of cells coalesce, the cell walls appear to break down and the plasma of the various cells intermingles, with the resultant production of regenerative granules varying in size from very minute bodies, scarcely discernible with the oil immersion lens, to larger forms that are readily visible when stained. Neisser's blue is a good stain for showing up these granules. On emergence from the "symplasm" these granules grow into young azotobacter cells and reproduce by fission.

6. Varieties 1 and 2 in cultures up to fourteen days old on Ashby's agar produce large capsules; variety 3 produces only small capsules and variety 4 produces no capsules.

7. All four varieties are motile in young cultures on Ashby's agar or in Ashby's solution.

8. In cultures more than fourteen days old, large, spherical, thick-walled cells are common. In varieties 1, 2 and 4 these occur in irregular groups; in variety 3 they occur in tetrads and sarcina packets. These appear to be resting cells or arthrospores, as at this stage multiplication by fission appears to have ceased for the time being. On transference to fresh media these thick-walled cells germinate, the cell plasma emerging from the thick wall as a large rod which at once proceeds to multiply by fission; the young cells are motile.

9. Heat-resistant endospores are not produced.

10. Involution forms varying much in size and shape occur commonly in cultures more than fourteen days old, in Ashby's solution or on Ashby's agar at 25°C. They are particularly numerous when cultures are grown at 37°C.

11. Some involution forms appear to multiply to a very limited extent by a budding process.

PLATE 1

FIG. 1. AZO. 1. Smear from a twenty-four hour culture on Ashby's agar at 25°C., inoculated from a seven-days old culture in Ashby's solution in which rapid growth was occurring and many cells were disintegrating. Smear shows very young cells presumably developed from the regenerative granules dispersed by disintegrated mother cells. Central corpuscles, possibly nuclear bodies, are noticeable in most of the young cells and fission in all stages is apparent; cells were actively motile. Stained with saturated alcoholic gentian violet. × 1000.

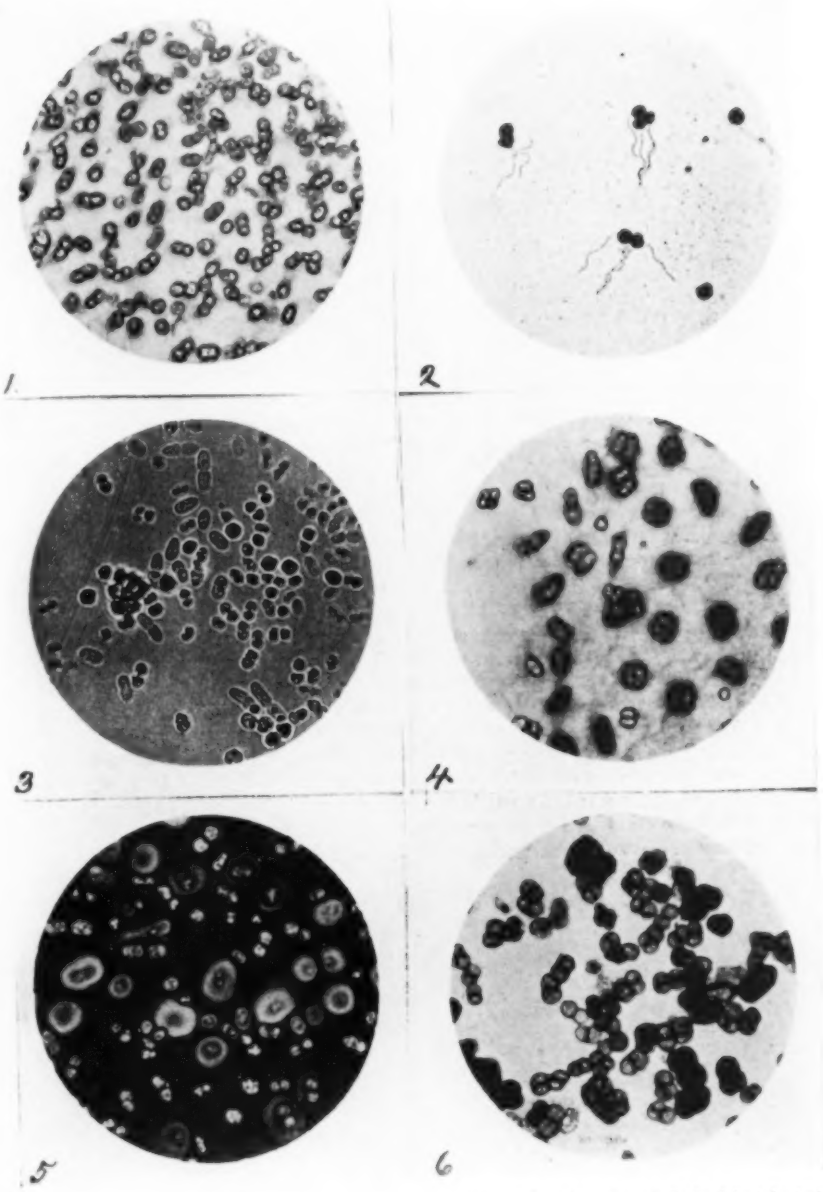
FIG. 2. AZO. 1. Cells showing flagella from same culture as figure 1, stained with Moore's modification of Loeffler's flagella stain. × 1000.

FIG. 3. AZO. 4. Resting cells or arthrospores germinating. Preparation in Lugol's solution from a twenty-four hour culture on Ashby's agar at 25°C., inoculated from a one-month old culture in which many resting cells were present. The dark, granular irregular spherical bodies are the resting cells and the light-colored rods are from the germinated resting cells. × 1000.

FIG. 4. AZO. 3. Smear from a four-day old culture on Ashby's agar at 25°C., showing fission first in one direction, then at right angles, giving tetrad forms common with this variety. Stained Neisser's blue. × 1000.

FIG. 5. AZO. 1. Smear from culture on Ashby's agar, six days old at 25°C., showing capsule formation and development of cells in many stages from granules dispersed from disintegrated mother cells. Granular mother cells also in evidence. Stained with saturated alcoholic gentian violet. × 1000.

FIG. 6. AZO. 2. Smear from culture fourteen days old on Ashby's agar at 25°C. showing resting cells or arthrospores. Stained with saturated alcoholic gentian violet. × 1000.



(Jones: Studies on Growth Cycle of Azotobacter)

PLATE 2

FIG. 1. Azo. 1. Smear from culture one month old in Ashby's solution, showing to the left two mother cells disintegrating, the liberated reproductive granules developing into short rods before dispersal. Stained with Heidenhain's iron hematoxylin. × 1000.

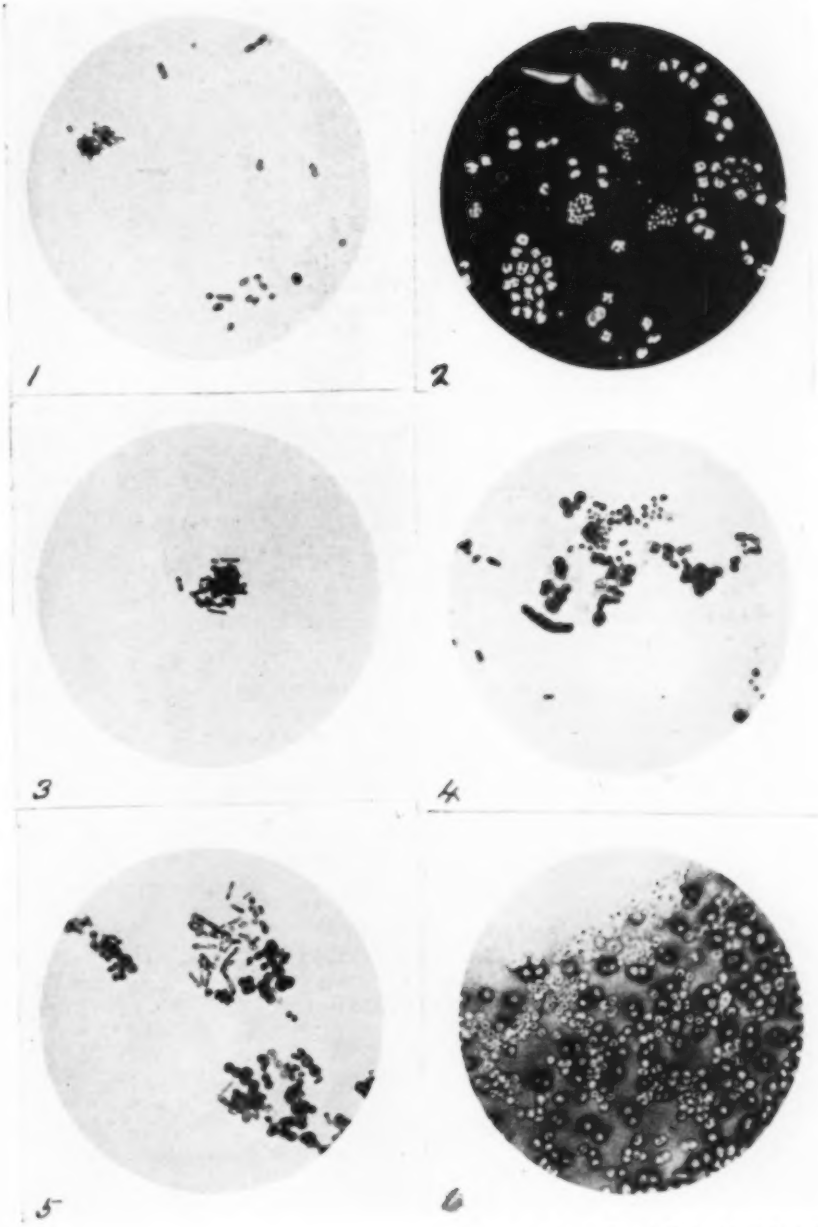
FIG. 2. Azo. 1. Smear from culture fourteen days old on Ashby's agar at 25°C., showing granular mother cells disintegrating. Stained with saturated alcoholic gentian violet which stains the capsule material, forming the background, leaving the organisms mostly negative to the stain. × 1000.

FIG. 3. Azo. 2. Similar preparation to figure 1 from culture of Azo 2 of same age. × 1000.

FIG. 4. Azo. 4. Smear preparation from a culture in Ashby's solution one month old at 25°C., showing various cell forms, particularly dispersed reproductive granules in various stages of development into young cells multiplying by fission. Stained with Heidenhain's iron hematoxylin. × 1000.

FIG. 5. Azo. 4. Similar preparation to figure 4 from culture one month old in Ashby's solution showing young rods and spheres developing from liberated reproductive granules, also small clusters of resting cells.

FIG. 6. Azo. 3. Smear from a twenty-hour old streak culture on Ashby's agar. Most of the cells shown are very young ones developing from dispersed reproductive granules and multiplying by fission. Stained with Neisser's blue. × 1000.



(Jones: Studies on Growth Cycle of Azotobacter)

PLATE 3

FIG. 1. Azo. 4. Smear from growth five days old on Ashby's agar at 25°C., showing various sized rods positive to the stain and granular spheres mostly negative to the stain, also a cluster of cells probably entering the symplastic stage described by Löhnis and Smith. Stained Heidenhain's iron hematoxylin. × 1000.

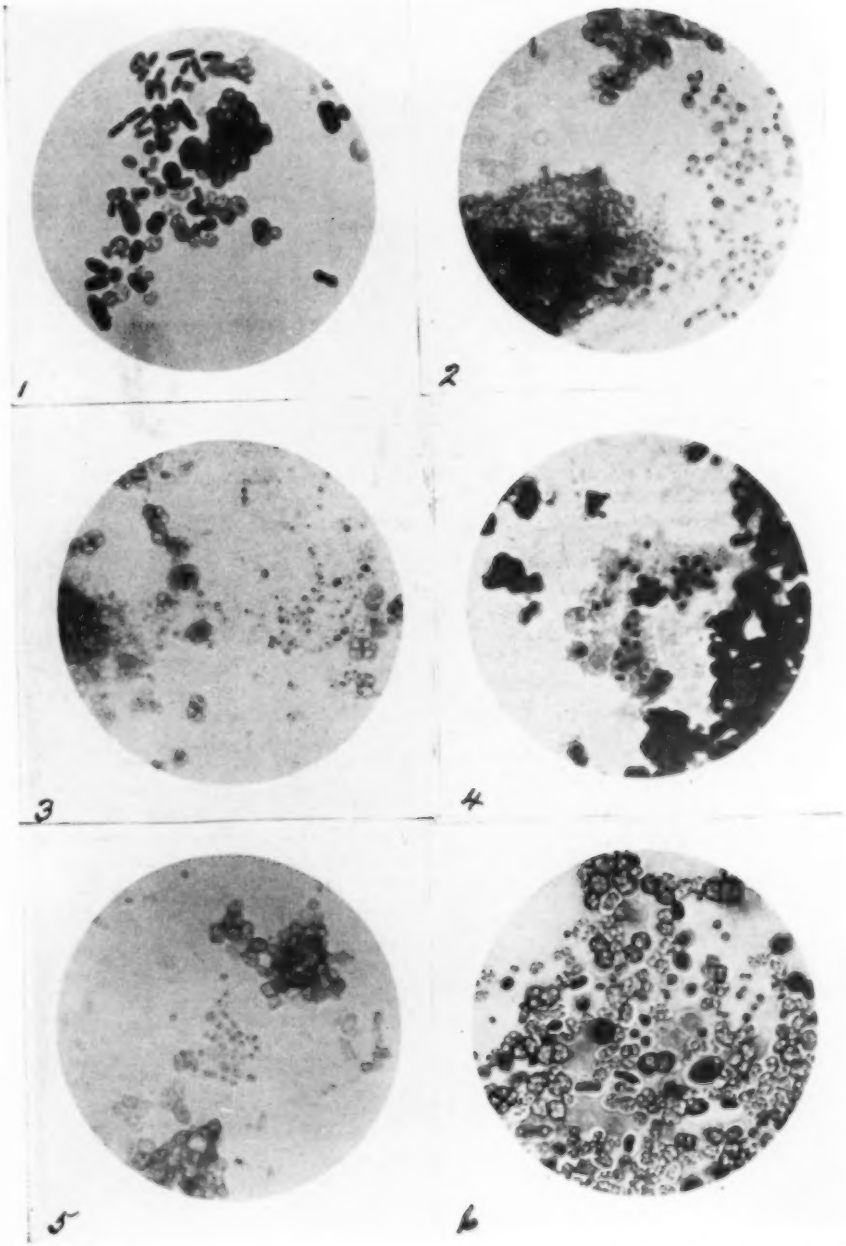
FIG. 2. Azo. 4. Smear from culture sixteen days old in Ashby's solution at 25°C., showing at the top a portion of a mass of cells entering the "symplastic" stage; at the bottom a portion of a mass of cells in an advanced "symplastic" stage where apparently the cells have fused and the reproductive granules are coming into evidence; at the right hand side a number of scattered young cells developed from reproductive granules liberated from another mass of "symplasm." Stained Neisser's blue. × 1000.

FIG. 3. Azo. 3. Smear from a streak culture three weeks old on a beef extract agar plate inoculated from an Ashby solution culture seven days old, showing a few tetrad forms of resting cells and to the left a portion of a "symplasm" in an advanced stage; to the right numerous minute reproductive granules escaped from the symplasm. Around each individual strongly stained granule is a zone of plasma negative or only slightly positive to the stain, clearly discernible in the smear but not showing up well in the photograph. Stained Neisser's blue. × 1000.

FIG. 4. Azo. 3. Smear from colony one month old on beef extract agar showing darkly stained clusters of resting cells also clusters of young cells developed from reproductive granules from disintegrated mother cells. Stained Kutscher's gentian violet; × 1000.

FIG. 5. Azo. 4. Smear from sixteen days old culture in Ashby's solution 25°C., showing two aggregations of cells probably entering "symplastic" stage and in the center a mass of young cells developed from regenerative granules. Stained Neisser's blue. × 1000.

FIG. 6. Azo. 3. Smear from a colony six days old on Ashby's agar at 25°C., showing granular mother cells disintegrating, dispersal of granules and various stages in development of reproductive granules into young cells multiplying by fission. Stained with saturated alcoholic gentian violet. × 1000.



(Jones: Studies on Growth Cycle of Azotobacter)

PLATE 4

FIG. 1. AZO. 2. Smear from culture seven days old on Ashby's agar 25°C., showing large irregular amoeboid cells, varying in density of protoplasm, also some granular forms; stained with Heidenhain's iron hematoxylin. × 1000.

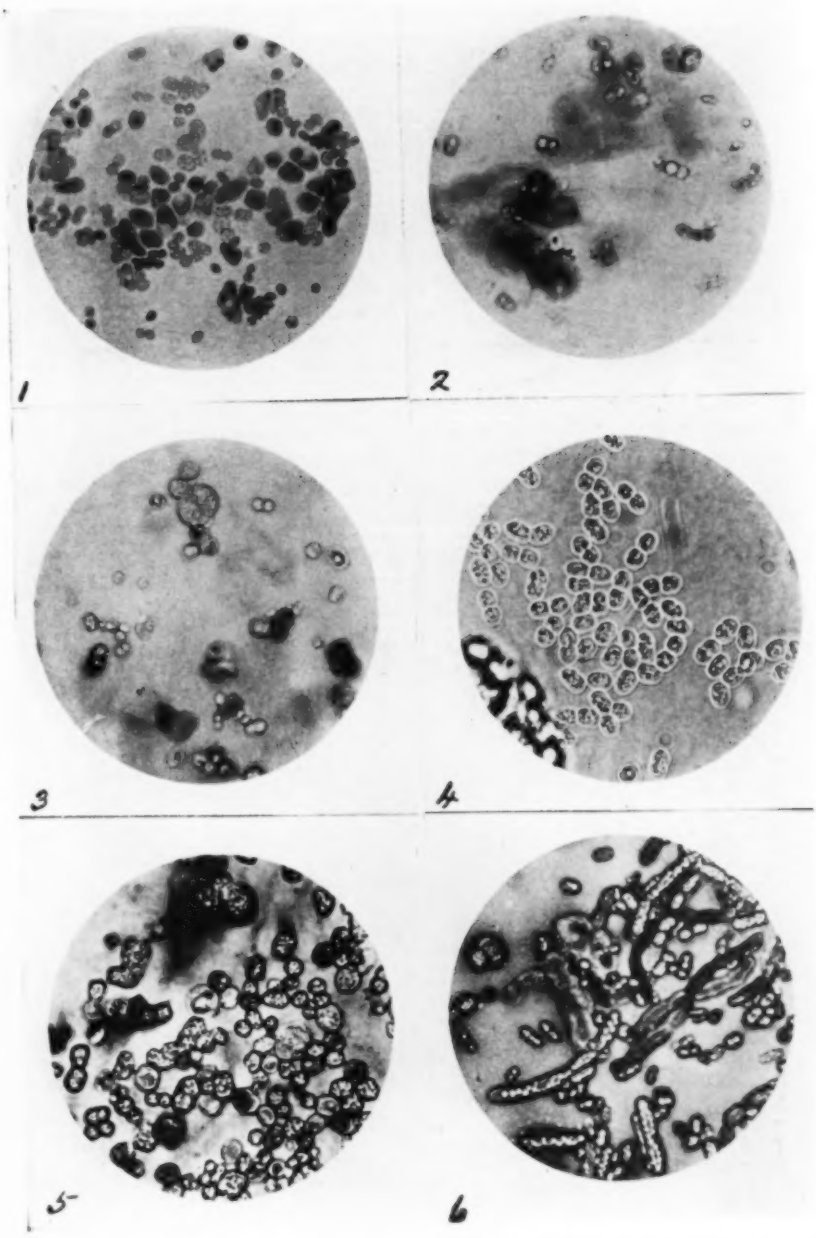
FIG. 2. AZO. 2. Smear from culture on Ashby's agar two days old at 37°C., showing darkly stained granules within the cells; stained Neisser's blue. × 1000.

FIG. 3. AZO. 3. Smear from culture on Ashby's agar two days old at 37°C., showing large granular cells apparently budding. Stained Neisser's blue. × 1000.

FIG. 4. AZO. 1. Large motile cells that were observed to break away from edge of colony four days old growing on agar hanging block in warm stage moist chamber, and swim around for twenty minutes or so in water of condensation accumulated at edge of colony. Photographed in living condition when quiescent. × 1000.

FIG. 5. AZO. 3. Smear from culture seven days old on Ashby's agar at 25° C., showing large granular cells and some involution forms with many small granules, positive to stain. Stained Neisser's blue. × 1000.

FIG. 6. AZO. 1. Smear from culture seven days old at 37°C. on Ashby's agar showing various elongated involution forms, some granular and some with homogeneous protoplasm. Stained with Neisser's blue. × 1000.



(Jones: Studies on Growth Cycle of Azotobacter)

THE DIAGNOSIS OF ANTHRAX FROM PUTREFYING ANIMAL TISSUES[1]

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Received for publication December 30, 1919

Of all infectious diseases of animals there is none easier to diagnose bacteriologically, when the material is fresh, than anthrax. In infected tissue, *B. anthracis* is always numerous and present in practically pure culture. This fact, together with the vigor of its growth on the simplest kinds of culture media, make its detection very simple. When the suspected material has undergone a certain amount of putrefaction, however, conditions are changed and difficulties arise. These difficulties are due to two things, viz., first, to the multiplication of a number of species of organisms which resemble the anthrax organism so closely as to cause difficulty in their differentiation; second, to the diminution in the numbers of the anthrax organisms present, this diminution being carried on progressively, as putrefaction proceeds, to the point of complete extinction.

The first difficulty has been satisfactorily overcome but the second cannot be, so long as putrescible tissues are shipped for considerable distances before examination. In such tissues the anthrax organism is usually prevented from sporulating because of lack of sufficient oxygen. In this stage it is quite rapidly destroyed by putrefaction, and, undoubtedly, in a considerable number of positive cases, the evidence is destroyed before the tissue is examined. Methods of sending material which would provide favorable conditions for the anthrax organism to sporulate as well as to reduce the moisture to a minimum, thus preventing the growth of saprophytic organisms, are perfectly feasible and

[1] Presented before Society of American Bacteriologists, December 30, 1919.

practicable but for some reason the practice of submitting putrescible animal tissues for examination has become a habit. A small piece of cotton string soaked in blood or spleen juice, placed in a clean dry bottle and shipped to a laboratory would furnish excellent material for the bacteriologist. The string would slowly dry out allowing any anthrax bacteria which might be present to sporulate. The dry string would then remain in satisfactory condition for bacteriological examination for months. This method is not used, however, and even if used, in a small number of cases putrefaction would have to be dealt with because of the material having been taken from the body of the animal after putrefaction had begun.

My experience with the problem has been in a diagnostic laboratory where animal tissues are received from various parts of the state of New York for examination. Many of the regions are somewhat isolated from the laboratory so far as rapid carrier service is concerned and in a considerable number of cases the material is not properly packed. As a consequence, we have considerable tissue to handle which has partially putrefied.

Tissues received for examination for anthrax consist mostly of blood, pieces of spleen, and ears. Of these, ears are received most often and are, on the whole, the most satisfactory tissue received. To procure an ear it is not necessary to open the carcass, thus spreading infection on the premises by liberating anthrax organisms if they are present, and since the ear contains so little soft tissue it will resist putrefaction much longer than other parts. If the weather is not too hot and the journey is not over twenty-four to forty-eight hours long, ears usually arrive in a satisfactory condition without ice. Many are received, however, which have been in transit for several days up to a week and these are always more or less putrefied. Blood samples, pieces of spleen and other organs almost invariably arrive in bad condition, if sent without ice (and this is frequent) and in not a few cases, even when well iced, because of having been delayed in transit.

Accompanying the specimen or frequently in advance of the arrival of the specimen, a telegram or letter is received requesting

a prompt reply. In many cases the health of other animals is at stake and sometimes it is the health of some of the people who have had to do with the care of the animal while sick or after its death. The element of time required in making a diagnosis is therefore of considerable importance.

LABORATORY PROCEDURE

A smear is first made from the sample of blood, spleen, pulp or blood from a subcutaneous ear vein as the case may be. This is stained with any of the common stains and examined immediately. In case the material is fresh and the case is positive the anthrax organisms will be found in abundance. In these cases the McFadyean reaction works beautifully. If the material has putrefied to any extent, organisms which closely resemble the anthrax bacterium are usually present. There may be several kinds of these organisms in a single sample. In addition to these there are nearly always present some larger organisms of a rod shape and having a slight resemblance to *B. anthracis*, and various organisms of other kinds. I have not had success with the McFadyean reaction in picking out anthrax organisms from such a mixture and the morphology can be taken as of no greater significance than as a suggestion of anthrax.

Cultures are made on plain agar plates. A small bit of tissue or a drop of blood is placed in a tube of bouillon and shaken. From this dilution, a loopful is smeared over the surface of one or more agar plates, using a bent glass rod as a spreader. If the material is relatively fresh and the number of bacteria does not appear to be great, the dilution is done away with, the plates being smeared directly. The cultures are incubated at 37°C. and examined after eighteen to twenty-four hours.

Many of the large organisms having some resemblance to *B. anthracis*, found in putrefying blood and tissue are anaerobes so that samples showing large numbers of these will usually show only a few *Bact. coli* or miscellaneous organisms on the plates. In these cases, a diagnosis of "Unable to find anthrax" is made. Injection of guinea-pigs does not yield any result as far as a diag-

nosis is concerned but the pig usually succumbs in from one to four days with a malignant oedema type of infection. The anthrax organism, if it was originally present, has been destroyed by the putrefaction.

In other cases, microscopic examination of direct smears having shown but very few anthrax-like organisms or none at all, when the culture shows only a few miscellaneous colonies, none of which resemble anthrax, a negative diagnosis is made without subjecting the material to a guinea-pig test.

A glance at the plates after incubation will serve to show a worker with any experience with the subject whether either anthrax or what I refer to as "anthrax-like" colonies are present. The most striking character of these colonies is their "ground glass" appearance, especially at the margins. To the unaided eye, the colony appears like frosted glass. The reason for this ground glass appearance may be readily appreciated when the colonies are moderately magnified. Just as ground glass has its well known opaque, whitish, velvety appearance because of numerous small facets the surfaces of which are facing in many directions and which reflect light from just as many directions as there are faces, so do these colonies have this appearance because they are made up of bacterial filaments arranged in parallel to form bundles which run in many directions and which reflect light as do the facets of the glass. The less the tendency of the organism to arrange itself in parallel chains the finer does the texture of the ground glass become. In colonies of *B. anthracis* the bundles are larger than in most anthrax-like colonies so the reflecting facets are larger and the texture of the ground glass is coarser.

The diameter of the colonies varies, depending on the number on the plate. When not crowded anthrax colonies will reach a maximum diameter of about 5 mm. If given plenty of room some of the anthrax-like colonies will identify themselves by spreading and becoming of much greater size than a true anthrax colony ever reaches. This is not always true, however, especially in a primary culture and the plates as made direct from the suspected material, do not always allow sufficient room for this growth to occur.

Stained preparations will serve to eliminate many of these colonies by showing organisms of a morphology differing sufficiently from *B. anthracis* to admit of a negative diagnosis. These differences are principally in greater length and the possession of ends markedly rounding. Stained preparations will by no means eliminate all of these organisms however for some show bacteria indistinguishable, as far as I am able to determine, from true anthrax. Hanging drops will also eliminate many of these organisms by demonstrating rapid motility and I have no doubt but that other tests would eliminate still others not detected by the methods given. These are all time consuming, however, and, as I have said, this is of importance.

An easy and rapid method has been devised which, during a period of over a year, has picked out readily all true anthrax cases (about 15 in number) from among a considerable number of negatives (about 70), the inoculation of guinea pigs being the criterion. This method consists simply in examining directly with a high power objective the minute structure of the suspected colony. When examined grossly or with low powers, these colonies are so similar as to make differentiation difficult in many cases; but when submitted to a greater magnification differentiation has been made with ease in all cases yet submitted to the test. Some of these differences are difficult to explain and can be appreciated only by actual trial. The method gives at once the morphology of the organism, whether or not it is motile, whether or not spores are present, their location and shape if present, and the relation of the organisms to each other in the colony.

The suspected colony is prepared for examination by laying aside the lid of the Petri dish and dropping a flamed and cooled cover glass directly upon its surface. If one edge of the cover glass is first touched to the surface of the medium at one side of the colony to be examined and then gently lowered with a pair of thumb forceps air bubbles will be avoided as there is sufficient water of condensation on the surface of the agar to fill the space beneath the cover-glass. A drop of immersion oil is now placed on the cover glass, the open plate placed on the

microscope stage and the edge of the colony carefully focussed upon with the $\frac{1}{12}$ mm. objective (fig. 1).

A true anthrax colony under these conditions shows beautiful festoons of parallel, closely packed filaments. The arrangement has been aptly compared to a carefully combed coiffure. Very seldom is the end of any filament seen at the colony margin; they appear to begin and end in the depths of the colony. A rather peculiar character, at first thought, is the fact that the filaments, unless examined very diligently, appear to be homogeneous and do not show the divisions between the individual organisms. I have not seen this character nearly so well marked in any of my anthrax-like colonies. It is undoubtedly due to the almost absolute squareness of the ends of the bacteria. In the case of other organisms which have ends slightly rounded and which form filaments, the individual elements are plainly shown because of the reflection and refraction of light from the curved surfaces of the ends of the organisms. My photomicrograph does not illustrate this character well for the camera seems to have detected the individual organisms much better than the eye is able to do.

The true anthrax colonies show few or no spores until after twenty-four hours when grown on 1.5 per cent beef infusion agar. This point aids in differentiating certain species which sporulate abundantly before this time.

The anthrax-like colonies show various characters which it would not be profitable to discuss here. At least half of these types prove to be motile organisms and are easily eliminated by this character. The motility is observed only at the extreme margin where a comparatively few organisms swim around actively. Many other organisms lie entirely free but with no signs of movement so it is probable that the motile stage is of very short duration. Certain observers have claimed that some organisms of this type were not motile in the primary culture but developed motility later in subcultures. This is probably explained by the observation above.

The arrangement of the filaments in none of the anthrax-like organisms has been exactly like that of the true anthrax. Fre-

quently the free ends of many of the filaments are seen projecting out from the margin of the colony, differing radically in this respect from the anthrax colony. The filaments are never so even and regular, or so homogeneous, or arranged in such smooth sweeping curves in the anthrax-like colonies as in true anthrax. The filaments are usually broken by sharp angular bends here and there, and in some cases they are very short and interlace.

Details of the many anthrax-like organisms encountered in putrefying material are too numerous to give here. To anyone who has a good picture of the true anthrax colony in his mind, it would be an easy matter to eliminate all of the "pseudo" forms which I have encountered and examined as I have described.

I do not advocate the method just described as a method to supersede entirely the use of guinea-pigs in the diagnosis of anthrax from more or less decomposed animal material, but I do believe it is a valuable aid in making a rapid diagnosis and will eliminate the guinea-pig in the majority of cases. In badly decomposed material, I believe cultural methods have certain advantages over the use of animals inasmuch as the anaerobic bacilli present in such material will frequently bring about death of the inoculated animal in less time than is ordinarily required for anthrax material to do this while these organisms offer no difficulties in the cultural method. Furthermore, it is known, in these cases, that the number of viable anthrax organisms are greatly reduced and it is conceivable that those which remain may be so attenuated in some cases that they may fail to infect the animals inoculated. The cultural method offers a small chance of detecting such cases should they occur.

SUMMARY

1. Many anthrax like organisms are encountered in the examination of partially putrefied animal tissues for *B. anthracis*.

2. The McFadyean reaction has not proven of great service in distinguishing the true anthrax from these contaminating forms.

3. Guinea-pig inoculation frequently fails because of the death of the animals from a malignant oedema-like infection before any anthrax organisms which may be present have time to develop.

4. The chief difficulty in the way of a cultural diagnosis of anthrax in these cases, the difficulty in distinguishing, rapidly, between the true anthrax and the anthrax-like organisms has been obviated by a method here given.

EXPLANATION OF PLATE 1

FIG. 1. MICROSCOPE, WITH OPEN PETRI DISH IN PLACE FOR DIRECT EXAMINATION OF COLONY

FIG. 2. ANTHRAX COLONY × 20

The black line at one margin is extraneous matter which fell on the open plate while it was being photographed.

FIG. 3. MARGIN OF ANTHRAX COLONY × 100

FIG. 4. MARGIN OF ANTHRAX COLONY × 600

FIG. 5 MARGIN OF AN ANTHRAX-LIKE COLONY × 600

FIG. 6. MARGIN OF AN ANTHRAX-LIKE COLONY × 600

Fig. 1

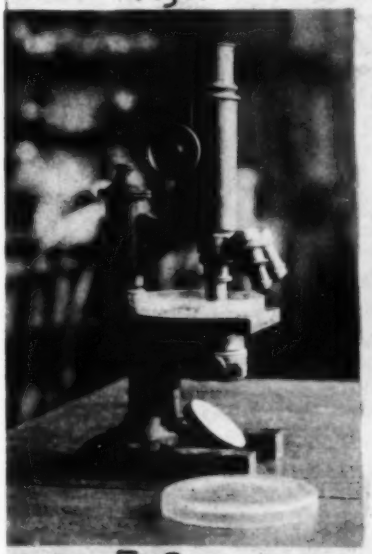

Fig. 4

Fig. 2

Fig. 5

Fig. 3

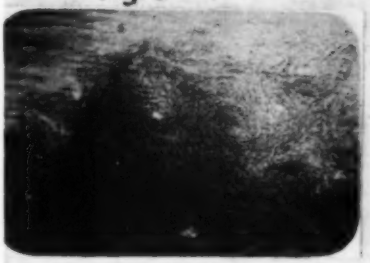

Fig. 6

(Hagan: Diagnosis of Anthrax)

BACTERIAL DECOMPOSITION OF SALMON[1]

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A study of the decomposition of the food fishes presents an interesting field for both the chemist and the bacteriologist. While the chemistry of fish decomposition has been investigated to some extent, comparatively little has been reported regarding the bacteriology of the problem. Browne (1917) reported the results obtained from his work on the decomposition of various fish during storage in ice stating that autolysis, rather than bacterial action, seems to play the most important part in the initial stages of decomposition. The part played by bacteria in the decomposition of sardines has been studied and reported by Obst (1919). The bacteriology of canned or preserved fish has received the attention of several workers. The question of whether or not the *Bacterium coli* is an inhabitant of the intestines of fish has been investigated by Browne, (1917), Eyre (1904), Houston (1903–04), Amyot (1901), and others but the papers of Browne and of Obst already mentioned seem to be the only studies made of the part played by bacteria in the actual decomposition of the flesh of fish before preserving.

The examination of a large amount of canned salmon at the Bureau of Chemistry during the past year has led to an extensive study of the raw fish from the time it is caught until it is put into the cans. The object of the investigation, from the bacteriological viewpoint, was to determine whether or not bacteria

[1] Presented before American Society of Bacteriologists, December 30, 1919.

[2] Published by permission of the Secretary of Agriculture. In carrying on this investigation valuable criticism and suggestions have been given by Dr. Charles Thom of this laboratory.

were responsible for the decomposition of the salmon and, if such was found to be the case, to establish a correlation between the physical appearance of the fish and the presence of the bacteria after holding the salmon for known lengths of time under known conditions. This necessitated the determination of total counts, and later of the kinds of bacteria, present in the muscular tissue of the salmon and also an investigation as to how these bacteria gain access to the flesh.

Four species of salmon were used in the investigation, namely, the sockeye or red salmon (*Oncorhynchus nerka*), the humpback or pink salmon (*O. gorbuscha*), the silver or coho salmon (*O. kisutch*) and the chum or dog salmon (*O. keta*). All the fish used were caught in fish traps near the San Juan Islands in Puget Sound. The fish were handled as they are handled commercially by the fishermen in that locality and accurate data were kept as to the methods of handling, the length of time out of water, the temperature at which they were held, etc.

The method of procedure was usually as follows: A trip was made to the trap late in the afternoon and the night was spent on the fishing boat alongside the trap. Early the next morning the trap was lifted and emptied of fish. One or two fish were selected for immediate examination and the others were placed in the hold of the boat to be transported to the fish house. On arrival at the fish house the desired number of salmon were placed in a large box in a corner of the fish house. A thermograph was kept with the fish throughout the whole period. Each morning one fish was removed from the box and examined. The general appearance of the fish was noted and recorded. When the odor and appearance of the salmon indicated that the fish were in an advanced stage of decomposition the experiment was terminated and a new experiment begun. Whenever it was found impossible to visit the trap on the fishing boat the fish were received at the fish house and the data in regard to the time and place of the catch were obtained from the fisherman. The fish usually arrived about eight hours after they were taken from the water. In the case of sockeye and humpback salmon three separate catches were allowed to decompose and were studied. The first

lot in each case was a preliminary experiment and the bacteriological examination was not usually conducted on more than the first one or two days. Only one lot of silver and chum salmon was examined. The temperature on the days on which the fish were held never fell below 50° F. nor rose above 70° F.

In the bacteriological examination of the fish total counts of bacteria were made from the muscle tissue of the back and belly of the fish and agar slant cultures were made from various parts and organs of the fish including the mouth, gills, stomach, ceca, intestines, heart, liver and kidney. Cultures in lactose broth fermentation tubes were made from the stomach, ceca, and intestines.

Since the work was necessarily often done in the field at considerable distances from the laboratory, some difficulty was experienced in the plating of the muscular tissue for total count but the technic used, in general, worked very well. The body of the fish was thoroughly washed with alcohol and the alcohol burned off. With instruments sterilized by flaming in alcohol a small flap of skin just posterior to the dorsal fin was carefully lifted and pinned back. A piece of muscle weighing approximately one gram was transferred to a sterile flask of known weight. The flasks used were of thick-walled, heavy glass in order that they might not break under the vigorous shaking necessary to break up the tissue. Known amounts of sterile, broken glass and sterile NaCl solution were added and the whole vigorously shaken until the tissue was thoroughly broken up. This suspension of tissue was diluted and plated according to the usual methods. The flask containing the remaining suspension was tightly stoppered and saved until the laboratory could be reached when it was weighed and the exact amount of original tissue computed. The sample of flesh from the belly was taken in the same manner just posterior to the ventral fin. Glucose agar was used and all incubations were made at room temperature.

The results of the experiments in determining total count are given in tables 1, 2, 3, and 4.

TABLE 1

LENGTH OF TIME OUT OF WATER	PART OF FISH EXAMINED	TOTAL COUNT OF BACTERIA FROM FLESH OF SOCKEYE SALMON		
		First series	Second series	Third series
hours		*per gram*	*per gram*	*per gram*
Within 2	Back	Sterile	Sterile	Sterile
	Belly	Sterile	Sterile	Sterile
24	Back	665	4,000	1,100
	Belly	5,750	8,000	8,000
48	Back		27,000	2,500
	Belly		36,000	15,000
72	Back		8,000,000	410,000
	Belly		12,750,000	920,000
96	Back		50,000,000	900,000
	Belly		155,000,000	6,400,000

TABLE 2

LENGTH OF TIME OUT OF WATER	PART OF FISH EXAMINED	TOTAL COUNT OF BACTERIA FROM FLESH OF HUMPBACK SALMON	
		First* series	Second† series
Hours		per gram	per gram
Within 2	Back	Sterile	Sterile
	Belly	Sterile	Sterile
24	Back	Sterile	120
	Belly	Sterile	1,420
48	Back	2,000	1,750
	Belly	37,000	1,700
72	Back	7,000	660,000
	Belly	50,000	1,600,000
96	Back	15,000	3,100,000
	Belly	60,000	3,500,000

* This lot of fish was thoroughly washed on arrival at the fish house. All blood and slime was removed.

† This lot of fish was left unwashed.

TABLE 3

LENGTH OF TIME OUT OF WATER	PART OF FISH EXAMINED	TOTAL COUNTS OF BACTERIA FROM FLESH OF SILVER SALMON	
		Washed fish	Unwashed fish
hours		*per gram*	*per gram*
Within 2	Back	Sterile	Sterile
	Belly	Sterile	Sterile
24	Back	Sterile	4,700
	Belly	200	71,000
48	Back	5,000	250,000
	Belly	6,500	480,000
72	Back	220,000	470,000
	Belly	2,500,000	2,200,000
96	Back	510,000	4,400,000
	Belly	2,800,000	11,600,000

TABLE 4

LENGTH OF TIME OUT OF WATER	PART OF FISH EXAMINED	TOTAL COUNTS OF BACTERIA FROM FLESH OF CHUM SALMON	
		Washed fish	Unwashed fish
hours		*per gram*	*per gram*
Within 2	Back	Sterile	Sterile
	Belly	Sterile	Sterile
24	Back	860	750
	Belly	2,100	18,000
48	Back	2,600	2,200
	Belly	130,000	28,000
72	Back	480,000	340,000
	Belly	500,000	2,300,000
96	Back	620,000	1,250,000
	Belly	1,180,000	3,410,000

Cultures from the mouth and gills of the fish were taken by simply inserting a sterile loop into these parts and then smearing the adhering mucus over the agar slant. After the material from the back and belly of the fish had been collected and the cultures from the mouth taken, the body cavity was carefully opened and the body wall cut in such a way that it might be pinned back, exposing the viscera. Organs from which cultures were to be taken were seared with a hot instrument and then cut slightly with sterile scissors. The sterile loop was inserted through the small opening and some of the blood and mucus transferred to the agar slant.

Since all the salmon examined were caught during their spawning migration, there was never any food found in the stomach. On rare occasions a small amount of partly digested food would be found in the intestine but for the most part the whole digestive tract appeared to contain nothing but mucus.

In handling one lot of humpback salmon the fish were washed thoroughly with running water, cleaning the bodies entirely of blood and slime. It was noticed that these salmon did not decompose as rapidly as had previous lots and in the subsequent experiments particular attention was given to the effect of washing fish as soon as they were brought ashore.

Examination of the mouths and gills of 41 salmon of various species has shown that microorganisms are always present even when the fish are examined immediately on being taken from the trap. Yeasts, bacilli of various kinds and cocci were usually found in large numbers.

Examination of the stomachs of 36 salmon has shown that no microorganisms are present in this organ during the first twenty-four hours, provided there is no feed in the stomach. In 3 out of 6 lots of fish examined, bacteria were found in the stomach after the fish had been held forty-eight hours and in one other lot the bacteria were recovered from the stomach after holding seventy-two hours.

In examining the ceca of salmon from 7 different lots, bacteria were found in but one lot when the fish were examined immediately on being caught. One lot showed bacteria in the ceca after

twenty-four hours, while in four other lots bacteria were not recovered from this part of the digestive tract until the salmon had been held seventy-two hours.

Living bacteria were found in the intestine of a salmon from one lot immediately on being caught. The intestine in this case contained some partly digested food. In 3 other lots bacteria were not found in the intestine until the fish had been out of water forty-eight, seventy-two and ninety-six hours respectively. In one lot no bacteria were found after the fish had been held for ninety-six hours.

In examining the heart's blood of 4 lots of salmon no bacteria were obtained when the fish was first caught. In 2 lots living bacteria were found in the heart's blood after twenty-four hours, in one lot after forty-eight hours and in the other lot after the fish had been held seventy-two hours.

Blood in the large vessels among the viscera was examined and no bacteria were found in the blood stream when the fish were fresh. In one lot bacteria were obtained from the blood stream after twenty-four hours and in the other lots they were found after forty-eight hours. It was noted that even at the end of ninety-six hours the blood in these vessels had not coagulated.

The kidneys of 6 lots of salmon were examined and in 3 lots no bacteria were found after ninety-six hours. In the other 3 lots living microorganisms were found after the fish had been held forty-eight hours.

The livers of 4 lots of salmon were examined and were always found to be sterile.

Direct smears from the parts examined and smears from the cultures obtained have shown that the organisms obtained from the viscera and the blood stream are at least morphologically similar to those present in the mouth and gills during the first twenty-four hours. These organisms have been isolated and are now being made the subject of a further study, the results of which will be presented in a later paper.

In regard to the physical appearance of the fish it was noted that during the first forty-eight hours, at the temperatures on Puget Sound, no marked decomposition takes place. The eyes of the fish remain bright; the gills are red with no foul odors; the

flesh is firm and sweet and the viscera remain normal in appearance. The fish at the end of the seventy-two hour period were in such a state of decomposition that they were designated as "stale." The eyes became slightly sunken, the gills dark in color and either sour or foul in odor; the digestive tract was darkened and usually rather foul-smelling. The flesh of the salmon at the end of the seventy-two hour period was usually soft with some sour or putrid odor. At the end of the ninety-six hour period the salmon were markedly decomposed and beyond the state where they should be considered as fit for food. The eyes were deeply sunken, the gills blackened and exceedingly foul smelling, the skin dry and cracked and the flesh very soft and putrid. The viscera of such fish were always very much darkened in color and very foul. As will be seen in tables 1, 2, 3 and 4, the total count of bacteria in the flesh from such fish in one case was as high as 155,000,000 per gram.

A study of the data in the tables has demonstrated several interesting facts. The muscular tissue of freshly caught salmon is sterile. In studying the intestinal contents of fishes, Eyre used pieces of flesh of the fish as controls and found them sterile. The counts obtained on the flesh from the belly are always higher than those from the flesh of the back. Since some of the bacteria in the flesh undoubtedly get there through the skin, the fact that the skin of the belly is thinner and more easily broken may help to explain this higher count. The very high counts in the flesh from both the back and belly are sufficient to explain the softening and decomposition of the tissue. In most cases the fish which were washed upon arrival at the fish house had lower total counts than those held unwashed. The washed fish did not decompose as rapidly as did the unwashed fish.

Results of the examination of the various organs of the body would seem to indicate that the sources of infection as regards these organs, and to a considerable extent as regards the muscular tissue also, are the gills and mouth. It would appear that a great many organisms make their way through the blood channels to the viscera and the muscular tissue within forty-eight to seventy-two hours after the fish has been removed from the water. Rough handling of the fish will, however, break the

skin and allow many organisms present on the surface to penetrate the flesh.

In regard to the presence of *Bacterium coli* in the digestive tract it may be briefly stated that in no case was this organism isolated from the ceca or intestines of the salmon examined.

SUMMARY

1. The muscular tissue of freshly caught salmon is sterile.

2. After ninety-six hours at temperatures between 50°F. and 70°F. the total count of bacteria in the muscular tissue has been found to be as high as 155,000,000 per gram. The high counts obtained are sufficient to explain the decomposition of the tissue.

3. Thoroughly washing the fish on arrival at the dock results in lower total counts. The washed fish decompose less rapidly than the unwashed fish.

4. The mouths and gills of salmon contain living microorganisms of various kinds even when fresh from the water.

5. The digestive tract of salmon is sterile when there is no food present. This is in agreement with the findings of Obst in studying sardines.

6. The various organs of the body become infected through the blood vessels usually within ninety-six hours after the fish are caught.

7. Salmon out of water more than forty-eight hours at temperatures between 50°F. and 70°F. are decomposed to such an extent that they are not desirable as food.

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THE FILTRATION OF COLLOIDAL SUBSTANCES THROUGH BACTERIA-RETAINING FILTERS[1]

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It has been noted by the writers that in the preparation of germ-free filtrates, notably in working with the anaerobic organisms, those media which contained raw meat pieces added aseptically were superior to media to which meat cubes were added and which were subsequently sterilized by heat. It was believed—a belief later confirmed—that the presence of the unaltered proteins was chiefly responsible for the superiority of those media of which they were an integral part. However, the addition of meat cubes to previously sterilized media without contamination thereof is difficult and unpractical on a large scale. Therefore, in order to supply proteins unaltered by heat the juice obtained by subjecting finely ground and previously frozen and thawed raw meat was suggested as a substitute for meat cubes in the expectation that this juice could be sterilized by passing through a bacteria-retaining filter.

Filtering of such a colloidal substance was found to be exceedingly difficult. Inasmuch as no literature seemed to be available which offered any assistance it is believed that a report of the technique now used by the writers will prove helpful to others. Preparation of the colloidal substances for the bacteria-retaining filter by preliminary passage through cotton, asbestos wool and filter paper does not appreciably enhance their filterability. However, by adding a sufficient amount of pulverized kieselguhr to the meat juice to make a very thin paste and pouring this mixture over a coarse filter paper, the filtrate so obtained will

[1] Presented before Society of American Bacteriologists, December 29, 1919.

possess a temporary filterability through the bacteria-retaining filter candle, but the lapse of any appreciable time between the preliminary and final filtration permits a reversion of the material into the original state of non-filterability due probably to coagulation of the proteins. Unfortunately, the time required in the accomplishment of this preliminary kieselguhr filtration is sufficiently long to defeat its own purpose and for this reason the kieselguhr mixture is poured directly over the filter candle omitting the paper filtration entirely. The meat juice as it comes from the press is cleared of the coarser particles by centrifugalization. A small amount of kieselguhr is then added to the clarified meat juice and this mixture again centrifuged. After the supernatant fluid is drawn off it is again mixed with a sufficient amount of kieselguhr to make a rather thick mixture, approximately the consistency of butter-milk or a thin gruel. It is this mixture that is poured in direct contact with the filter candle. The best results will be obtained by using a minimum amount of vacuum. By placing the filter candle upright in a mantle, gravitation will materially assist in minimizing the required amount of vacuum necessary to draw the material through the filter candle into the vacuum flask.

In the event that the filtration process cannot be carried out immediately after the expression of the meat juice, some coagulation of the proteins is apt to take place, which should be corrected by centrifugalization just before filtering. It has been noted that the finished product after passage through the bacteria-retaining filter will, on standing or by application of heat, coagulate just as completely as the meat juice that has not been subjected to the filtration process.

By this process, meat juice, milk, hemolized blood corpuscles, etc., may be filtered at a rate which will compare favorably with the filtration of crystalloidal solutions and bouillons.

NOTES ON THE FUSIFORM BACILLI OF VINCENT'S ANGINA

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There is still some difference of opinion expressed in standard texts as to the Gram-staining qualities of the fusiform bacilli found in Vincent's angina and allied conditions. In Hiss and Zinsser it is stated: "Stained by Gram, they are usually decolorized, though in this respect the writers have found them to vary." Stitt calls this bacillus Gram-negative but states that it "is not markedly Gram-negative." No attempt will be made to review completely the literature on this point, but a few statements may be quoted to show that there is such disagreement.

A good summing up of the literature is that of Beitzke (1904) and many of the opinions quoted here are from this article, as well as from those of Weaver and Tunnicliff (1905, 1907) and of Babes (1906).

Weaver and Tunnicliff, from a large variety of material, found the bacilli to be Gram-negative, though they state that, unless thoroughly decolorized by alcohol, the cells retain a very faint bluish color. They mention (1905) that others have found longer decolorization to be necessary; that Vincent, Abel, and Niclotte and Marotte found the bacilli Gram-positive, but that all other authors contradict this. Vincent in his original article on Hospital Gangrene (1896) describes the bacilli as Gram-negative. In a later article (1899) on the anginas, he also describes them as Gram-negative (p. 613) and I can find no reference to them as Gram-positive, such as Weaver and Tunnicliff (1905) (p. 449) cite. Babes (1906) found fusiform bacilli from the gums in scurvy Gram-negative and states that Bernheim and Pospichill, in 30 cases of angina and other conditions found them Gram-negative.

He mentions Veullar and Zuber's having found such bacilli Gram-positive in material from the appendix. An article by Veillon (1898) describes *Fusiformis* from the appendix as Gram-negative (p. 540) and I could find no description of it as Gram-positive in his articles.

In summing up some of the literature on noma, Weaver and Tunnicliff (1907) mention that Foote, Elder, Blumer and MacFarlane, Hofman, Buday and Nicolaysen all found fusiform bacilli Gram-positive, while in their own case of noma they were Gram-negative, "except in the dark spots," though they held the stain longer than the spirilla also present. In some of the last quoted articles, the description does not sound typical of Vincent's organisms and in most no spirilla were present with them. Dick (1913) in 7 post-mortems of meningitis, cerebellar abscess, gangrene of the lung, pneumonia, empyema, and peritonitis, found the fusiform bacilli always Gram-negative. The same was true of the 3 strains which were cultivated. Krumwiede and Pratt, (1913, a, b) of 15 strains from the throat and teeth, found all Gram-negative, in smear and culture. Tunnicliff (1906) who cultivated them, found the fusiforms Gram-negative in cultures.

Altogether of 17 authors or groups of authors who mention the Gram-stain, 6 found the fusiform bacilli Gram-positive, 8 found them Gram-negative, while of the 3 remaining, Foote found them Gram-positive but that care was necessary to avoid decolorizing too much, Hofman, Gram-positive but "care was necessary in differentiation" and Bernheim and Pospichill found them Gram-negative after longer decolorization.

This disagreement might be due to the fact that entirely different organisms of fusiform appearance were present in different cases. In view of the large number of cases of Trench Mouth that we were examining it seemed worth while to test a series of smears against standard Gram staining technique. Unfortunately this was not undertaken while the press of work was on, so records are available for a relatively small number of cases. Only cases which showed a profusion of both bacilli and spirilla were included. Twenty-four examinations on 18 cases could thus

be included, examinations being repeated on 4 of the cases, and three examinations being done on the other case. In 17 examinations, 2 standard methods of staining were used on each, the old long method (one minute carbol gentian violet, two minutes Gram's iodine, five minutes alcohol, one minute safranin) and the newer shorter method (fifteen seconds' gentian violet, washing off twice with Gram's iodine, fifteen seconds' washing with alcohol, one minute safranin). Of the other 7 examinations, the short method was used on 6, the long method on the other. Of the 18 cases, smears were taken in 14 from lesions of the throat or tonsils, in 4 from lesions of the gums.

Of the 24 examinations, the bacilli were found to be markedly more numerous than the spirilla in 17, while the spirilla were equal in number or more numerous in 7. In the cases where examinations were repeated, these relations remained constant in 4, while in the other case the bacilli were first more numerous than the spirilla, and five days later the spirilla were found to be more numerous.

The staining qualities were found to vary markedly with both methods. In the main, the two methods checked, Though in some examinations a larger number of bacilli were Gram-positive by one than by the other method, a majority usually stained in the same way by both methods, in any smear. There was sufficiently marked disagreement to make it questionable which were most numerous in only three examinations. As a check on the methods, it was noted that the streptococci nearly always present in the same smears were always markedly Gram-positive, while the spirillae were invariably Gram-negative by both methods.

Of the 24 examinations, in 12 over half the bacilli were Gram-positive by one or both methods. In 9 over half were Gram-negative. In 3 others, there was disagreement, 2 showing a majority Gram-positive by the short and Gram-negative by the long methods, while 1 showed the reverse relations. Of the 17 in which both methods were used, 7 showed a majority Gram-positive by both methods, 7 a majority Gram-negative by both, while in 3 there was the disagreement noted above. In the 5 cases where repeated examinations were done, the staining

varied as follows: In 4 a majority were Gram-negative at first but examination after two to five days showed a majority Gram-positive. In 1, the majority remained Gram-positive at first and second examinations. The organisms varied greatly in length, and it was thought some difference in staining coincident with the difference in size might be observed. While the longer thread-like organisms which were considered as involution forms, were almost always Gram-negative, the typical fusiforms, of whatever length, showed about an equal number of cells Gram-positive and Gram-negative.

A few attempts were made to cultivate the organisms, to observe whether the Gram-staining properties changed with age. Cultures were made from gingival ulcers, or from the tonsils, on Loeffler's serum made anaerobic by the Büchner-Wright method, also in anaerobic meat-broth and in Veillons. It was found that in the first and second cultures a fair number of slender bacilli could be found, some of which had a distinctly fusiform appearance. The majority of these were Gram-negative, a few Gram-positive. No spirilla were ever found.

One strain from an ulcerated throat showed a good many distinctly fusiform oganisms in the third generation after subculture in aerobic and anaerobic meat-broth for fifteen days. It is interesting that this strain, which in direct smears and first cultures showed a majority of Gram-positive bacilli, showed a much larger number Gram-negative in the second and third generation. In the older growth of the original culture this tendency was even more plainly shown. The aerobic meat-broth after ninety-six hours showed the bacilli all Gram-negative, while in the anaerobic meat-broth about half were Gram-negative. This was the only strain in which such a tendency to Gram-negativeness in well preserved organisms from cultures could be definitely made out. (In several old cultures of the strains showing a few bacilli most were Gram-negative, but a large number of these cells had lost their definite outlines, stained feebly and were considered dead or moribund.) These cultures all had a foul odor.

In one post-mortem an organism was found which from its source and morphology must be considered as at least very closely allied to the bacillus of Vincent. The case was one of death from broncho-pneumonia following a large abscess of the jaw directly continuous with the root of an ulcerated tooth. Smears from the ulcerated area, the abscess, the larynx, the large and small bronchi, all showed a profusion of fusiform bacilli, morphologically indistinguishable from those found in Vincent's Angina. A few ordinary pyogenic cocci were found in the smears but the bacillus was the predominant organism. No spirilla were found in any smears and none of the spore-bearing bacilli to be described. In the direct smears, about half the fusiforms were Gram-positive and half Gram-negative.

In all the aerobic broth cultures from these regions a few fusiform bacilli were found; in some a fairly large number. All showed also many cocci, which proved to be *Streptococcus viridans* and a hemolytic staphylococcus. In a culture from the larynx made on Loeffler's blood serum, the largest number of fusiform bacilli were found, and the fusiforms had greatly increased, relatively to the cocci, after five to six days. Aerobic plates made from this culture showed in twenty-four hours large numbers of peculiar small dew-drop colonies with moist, spreading edges. Second plates from these colonies gave nothing but such colonies appearing like pure cultures. Smears from well isolated colonies, however, while they showed many fusiform bacilli, showed also a club shaped, Gram-positive spore-bearer. On referring back to the original Loeffler's tube a few of these spore-bearers were also found. Cultures from these colonies in aerobic and anaerobic meat-broth and plain broth showed at first nothing but fusiform bacilli, but later in all these cultures, spore-bearers developed. One culture after sixteen days showed only a very few spore-bearers and many perfectly typical fusiform bacilli. Transfers back on plates gave the same colonies with the same mixture of fusiforms and spore-bearers. These transfers from liquid media to plates and back again were repeated as far as the seventh generation with the same results. Orders to the states interrupted the work before the fusiform bacillus could

be isolated from the spore-bearer. It seemed possible that the spore-bearers were a form of the fusiform bacilli appearing when it was grown aerobically and especially on solid media. But remembering that Weaver and Tunnicliff and also Abel found the same intimate relation of the bacilli to the aerobic cocci in isolated colonies, it seemed more likely that the spore-bearer was another organism present in the larynx or as a contaminant in the culture, whose aerobic growth furnished the conditions necessary for the growth of the more anaerobic fusiform bacillus. With regard to the staining characters, the fusiforms in the cultures varied markedly and with no ascribable relation to the conditions present. The question of the pathogenicity of this strain was not determined by animal experiment. Though found in such profusion in the lesions, it may have been only a secondary invader from the teeth following streptococcic infection.

In summing up, it may be said:

1. Bacilli were found to be more numerous than spirilla in 17 out of 24 examinations.

2. The fusiform bacilli of Vincent's angina show much greater variation in their Gram-staining properties than ordinary organisms. Where there were any number of bacilli present, smears were never found which did not show both Gram-positive and Gram-negative bacilli in the same smear. Many were found in nearly all smears which were Gram-negative with Gram-positive granules, most frequently 4 granules in a pair of bacilli, but this number varied. These Gram-positive granules seem to be one of the most constant mophological characteristics of the organisms, almost as constant and distinctive as the polar bodies in *Mycobact. diphtheriae.*

3. A slight predominance of Gram-positive organisms was noted, but not great enough to justify any conclusions.

4. Method of staining is not responsible for the variation noted.

5. The fusiform bacilli may be cultivated anaerobically or aerobically when other aerobic organisms are present. They could not be isolated by our methods, though others have succeeded in doing so.

6. No spirilla were found in cultures.

7. An organism greatly resembling the fusiform bacilli of Vincent's angina was found as the predominant organism in the post-mortem on a case of broncho-pneumonia. It was grown to the seventh generation, but not isolated.

My thanks are due to Captain F. S. Perrings, M. C., in charge of the Laboratory, Evacuation Hospital 19, for obtaining the post-mortem material, and for his coöperation at all times.

Since this paper was finished, I have found fusiform bacilli very like those of Vincent's angina occurring in two unusual locations. One was in smears from a peculiar ulcer surrounding the lower canaliculus; the other in cultures from the pus of a unilateral chalaziosis in which both lids were very extensively involved. In the second case it appeared in pure culture and grew well aerobically on the ordinary media. In both cases it may have been a secondary invader but it is interesting to know that such an organism may inhabit the conjunctival sac and Meibomian glands, a fact not previously recorded, so far as I know. The author hopes to report further on these cases.

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A HIGHLY RESISTANT THERMOPHILIC ORGANISM

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An examination of spoilage samples of "Standard Maine Style" corn which had been packed in the usual manner and processed at 118°C. for 75 minutes showed the presence of a thermophile. The same organism was later found in spoiled cans of string beans and corn on the cob. It appears to be an unknown species and the name *Bacillus stearothermophilus* is proposed for it.

Its cultural characteristics are as follows:

Name proposed: B. stearothermophilus. (N. S.)

Source: Canned corn.

Date of isolation: October 3, 1917.

Vegetative cells: Medium used, agar; temperature, 60 to 65°C; age, twenty-four hours; form, large rods; arrangement, majority single, some in pairs end to end and few in chains of three or four. Size of majority 0.8 by 3.5 micra; ends rounded.

Relation to oxygen: Aerobic, facultative anaerobic.

Endospores: Present; location, polar. Size of majority 1 by 1.5 micra.

Motility: None.

Flagella: None. Muir's modification of Pittfield's method, and Loeffler's method.

Gram stain: Negative.

Nutrient broth: Surface growth, none; no pellicle or ring; clouding of medium uniform, odor, absent; sediment, compact, abundant.

Agar stroke: Growth, moderate; form of growth, generally filiform, sometimes slightly beaded, never spreading; elevation of growth, quite regular; optical character, translucent; chromogenesis, none; color, dirty white; odor, absent; consistency, butyrous.

Agar colonies: Growth moderate; temperature 60° to 65°C. form, circular; surface, smooth; elevation, flat; edge, regular; maximum diameter observed 2 mm., white opaque spot in center surrounded by several concentric rings.

Gelatin: Temperature 20°C., no growth; 60° to 65°C. growth but no liquefaction. (Inoculated tube showed uniform clouding after incubation for three days, solidified when placed in ice-box.)

Glucose agar stroke and stab (litmus): Growth, acid throughout.

Lactose agar stroke and stab (litmus): Growth, acid throughout.

Sucrose agar stroke and stab (litmus): Growth, acid throughout.

Potato: Growth, none.

Potato starch agar: Growth copious.

Corn infusion (litmus): Strong acid after twenty-four hours. Starch digested.

Temperature relations: Optimum temperature for growth 50°C. Maximum 76°C. Minimum 45°C.

Litmus milk: Growth begins after twenty-four hours, acidified, litmus partly reduced. No coagulation after forty-eight hours. After four days litmus completely reduced and casein digested, leaving heavy sediment in bottom of tube. (Control tube showed loss of color only after six days, no coagulation, no digestion.)

Indol production: Absent.

Nitrate broth: Not reduced; ammonia, none.

Glucose broth: Gas production, none.

Lactose broth: Gas production, none.

Sucrose broth: Gas production, none.

The thermal death relations at two temperatures and in media containing different numbers of spores are as follows:

MEDIUM	pH VALUE	TEMPERATURE	SPORES PER CUBIC CENTIMETER	TIME TO KILL
		°C.		
Corn broth	6.1	100	12,500	17 hours
Corn broth	6.0	120	50,000	11 minutes

THE BIOLOGY OF CLOSTRIDIUM WELCHII

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I. INTRODUCTION

The organism causing gaseous phlegmon or emphysematous gangrene was discovered by Welch and Nuttall at an autopsy in 1892 and was named by them *Bacillus aerogenes capsulatus*. In the revision of the nomenclature of bacteriology it was given its present name, *Clostridium Welchii*.

Many investigators have studied this organism and have proposed different theories to explain the symptoms following infection. During the late War, cases of wound infection by *C. Welchii* were reported among the French and British soldiers on the western front. Such infections have frequently led to disastrous results, terminating in death and needless amputation owing to lack of knowledge regarding the causes of the local destruction of tissue.

In 1917 Bull and Pritchett discovered that *Clostridium Welchii* produced a soluble toxin which was capable of causing the lesions characteristic of infections by this organism and which was neutralized by a specific immune serum. These facts have been confirmed by this investigation which was carried on in 1916, 1917, and 1918, to study the biology, pathogenicity, and distribution of the gas bacillus.

II. ISOLATION

The media selected for the isolation of *Clostridium Welchii* were glucose broth, glucose-liver broth, glucose-liver-agar and milk. The liver broth and agar were prepared by the method given in Standard Methods for Water Analysis 1912. Ordinary market milk adjusted to + 1 to phenolphthalein was used. Before sterilization, the surfaces of the media were covered with a layer of paraffin oil (albolin).

Pure cultures were obtained by inoculating tubes of sterile milk, previously covered with oil, with the material supposed to contain *Clostridium Welchii* and heating to 82° to 85° for fifteen minutes. The tubes were then cooled to 37° and incubated at that temperature. The presence of the bacillus in market milk was determined by transferring small portions of the milk to other sterile tubes; or the tubes of market milk were heated directly to 82° to 85° for fifteen minutes, cooled to 37°, and after covering the surface with sterile paraffin wax, incubated.

A positive reaction showed "stormy fermentation," i.e., coagulation of the casein, copious formation of gas, fragmentation of the curd, derangement of the cream layer and a detectable odor of butyric acid. However, milk cultures sometimes contained gas bacilli and yet failed to show this typical reaction due to various reasons. Cultures which showed a positive reaction were inoculated into tubes of glucose-liver broth and incubated from four to six hours, at which time the culture presented a "steaming" appearance. At this time plates were made on glucose-liver-agar, employing anaerobic technique, and incubated at 37°. After sufficient incubation (twelve to twenty hours)

typical *Clostridium Welchii* colonies were fished from around the gas zones and reinoculated into glucose-liver broth and later into milk. The purity of the culture was determined by microscopic examination. Although glucose agar and glucose broth with meat extract gave satisfactory results, liver medium was used in the greater part of this work, since *Clostridium Welchii* grew luxuriantly in the presence of glycogen.

The Welch-Nuttall incubation test was tried in order to prove that the "stormy fermentation" is characteristic of *Clostridium Welchii* and gave positive results throughout, with the recovery of the organism from the organs and body fluids of the animal.

III. DISTRIBUTION

Clostridium Welchii is extensively distributed in nature in the spore form. It has never been found in the vegetative form except in the tissues of the animal body. Members of this group have been isolated from the soil, dust in barns, dirt on laboratory floor, street dirt, from grains, such as gluten, meal, beet pulp and oats, from sewage and oysters, oyster liquor and mud surrounding the oysters.

Clostridium Welchii was present in most of the pasteurized milk examined in Providence, R. I., regardless of its bacterial content. Raw milk samples were consistently contaminated with spores of *Clostridium Welchii* in certain cases, while in other cases negative results were obtained. Clean milk contained *Clostridium Welchii*, although in less numbers and in fewer cases than milk of high bacterial content. Few dairies were continually free from *Clostridium Welchii* infection.

Clostridium Welchii was found on all parts of the cow, on the ceiling and floor of the barn, walls, cobwebs, milk utensils, milker's hands, barn dust and stable air. Milk direct from the cow was free from *Clostridium Welchii* and, therefore, became contaminated through some external source of uncleanliness and carelessness after the milk had been drawn.

Feces from human beings, guinea pigs (rarely), the cow, horse, rabbit, calf, hen, and dog contained spores of *Clostridium Welchii*

in considerable numbers. Two cans of preserved tomatoes packed by the cold pack method showed evidence of gas fermentation and when cultures were isolated anaerobically, *Clostridium Welchii* was found to be the cause of the fermentation. This organism therefore appears to have as broad a distribution as *Bacterium coli.*

TABLE 1

The number of cultures used in the subsequent study by sources

NUMBER OF CULTURES	SOURCE
56	Milk (pasteurized)
469	Milk (raw)
55	Human feces
18	Cow feces
18	Horse feces
10	Guinea-pig feces
626 total	

IV. MORPHOLOGY

1. Size and shape of organisms. *Clostridium Welchii* usually appears in cultures as a straight, plump rod, varying in size from 3 to 6 micra in length and from one to one and a half micra in breadth, according to the nature of the medium. The ends of the rods are slightly rounded or square cut. In old cultures the rods may be slightly curved or may form threads, filaments or long chains. In freshly isolated cultures they occur singly, paired, in clumps and sometimes in short chains of three to six bacilli, with the rods in a straight line or at an angle. Cultures grown for a long time on bloodserum and in liquid media containing coagulated egg-white, show long chains, rather slender threads and filaments, varying in length from 5 to 6 micra to threads extending across the microscopic field. Variation is more pronounced in the presence of a fermentable substance. An acid reaction is more favorable to variation than an alkaline reaction. The rods are more slender in gelatin and nutrient broth than in other media. In milk cultures the organisms are comparatively short, presenting a coccoid appearance. Greater

variation in length and thickness appears in old cultures than in those freshly isolated from the body tissues, although stained preparations from cultures made from body tissues, heart's blood and body fluid occasionally show very long chains and threads.

2. Capsules. Capsules were demonstrated in milk or glucose-liver-broth cultures made directly from body fluids or body tissues, from milk or feces, but cultures grown for any length of time on artificial media could not be made to form capsules unless reinoculated into the animal body or grown in milk. The capsule surrounds the bacterial cell and varies from one to one and a half micra in thickness. Welch's special stain was employed.

3. Staining. Clostridium Welchii stains readily with the ordinary dyes such as gentian-violet, safranin, methylene blue, carbol-fuchsin and eosin, either uniformly or with small unstained areas. In every case the bacilli from young cultures when stained by the regular Gram's method retain their color completely.

4. Motility. Specimens prepared from glucose-liver broth after incubation at 37° for periods varying from one to twenty-four hours do not show motility by the use of the usual hanging-drop method. No attempt was made to stain flagella.

V. SPORE FORMATION

1. Conditions governing sporulation. Sporulation takes place in the following media: Plain peptone water, Dunham's solution, peptone water plus a small amount of coagulated-egg-albumen made neutral or slightly acid, a solution of 5 per cent and 10 per cent coagulated-neutral-egg-albumen, a physiological salt solution plus coagulated-egg-albumen, nutrient gelatin and nutrient agar plus a small amount of coagulated-egg-albumen, plain liver broth, plain nutrient broth, plain nutrient broth plus coagulated-egg-albumen, blood serum, mannitol broth, mannitol-liver broth and a neutral suspension of feces. Strains of *Clostridium Welchii* vary in their ability to form spores in glycerol and inulin broth, with or without liver as a base instead of meat.

In market milk spores of *Clostridium Welchii* may enter as a contamination, but do not germinate except under very special conditions. Spores germinate rapidly in sterilized milk and remain in the vegetative stage without again forming spores. *Clostridium Welchii* has never been found to occur in nature in the vegetative form. This is undoubtedly due to its strictly anaerobic requirement and its inability to withstand an unfavorable environment. *Clostridium Welchii* never sporulates in the tissues, organs and body fluids of the living animals but does sporulate in the intestine.

Spores of *Clostridium Welchii* do not develop below 10°, and die out slowly at this temperature. Market milk, when heated to 80° to 85° for fifteen minutes and then kept on ice, shows no evidence of decomposition or fermentation due to *Clostridium Welchii*, but when allowed to stand above 20°, the characteristic "stormy fermentation" occurs in from twenty-four to seventy four hours. If the milk has not been heated to 80° to 85° for fifteen minutes, *Bacterium coli* and the other facultative aerobes multiply in excess of *Clostridium Welchii* and thus obscure its action, producing a peculiar non-characteristic digestion and decomposition of the milk.

2. Morphology of spores. Great irregularity is observed in the size, shape and location of the spores. They are usually oval, from 1.5 micra to 3 micra long and slightly thicker than the diameter of the bacillus. The spores form in the middle of the rod or slightly toward one end. They present, in preparations stained by Hauser's method, an unstained glistening appearance of a highly refractive character, while the ends of the organism stain deeply and completely.

3. Germination of spores. On germination, the spores show first a change in their refractive property; this is followed by elongation, with a final bursting through of the spore membrane at one pole and the outgrowth of the bacillus. Germination takes place in media containing glucose, galactose, lactose, maltose, sucrose, dextrin and starch. If there is a sufficient amount of the fermentable substance present, all the spores germinate. After all fermentable material is exhausted any

remaining spores fail to germinate and are observed microscopically. On the other hand, it has never been possible to cause sporulation of a vegetative strain in the presence of a fermentable carbohydrate.

4. Relation of spores to acidity. Spores of *Clostridium Welchii* withstand a wide range of reaction, surviving + 12 to − 2 per cent. Ordinarily spores are kept in neutral or slightly acid media but in some cases where all the spores failed to germinate in sugar media they could be recovered for a long time even if the acidity had reached a maximum of + 10 or + 12 per cent. Spores will develop in slightly acid or alkaline media but are formed best in neutral media. An environment of 5 per cent acid or 3 per cent alkali will inhibit the formation of spores but if already formed they will survive such an environment for a long time and can be preserved in such a condition. Media of − 2 reaction did not inhibit the formation of spores from the vegetative forms.

VI. CULTURAL REQUIREMENTS

1. Aerobiosis. Clostridium Welchii is an obligate anaerobe which requires strict anaerobiosis for its growth. All anaerobes, especially obligate anaerobes, have an optimum oxygen tension above which growth ceases. Although this tension for *Clostridium Welchii* is greater than for some others of this class, anaerobiosis must be fully maintained to obtain consistent and satisfactory results. Milk tubes which are not rendered strictly anaerobic before inoculation with this organism show either no growth or simple coagulation without a trace of gas formation, presenting that atypical reaction so often reported by investigators. This same phenomenon is observed with stock media which has been sterilized under a film of oil (albolin).

At the end of three months, the oil does not admit oxygen in a sufficient quantity to inhibit the growth of *Clostridium Welchii* when inoculated. At the end of five months, however, oxygen has been absorbed by the medium to such an extent that the optimum aerobic supply is exceeded and consequently growth

does not occur on inoculation. By using media containing different percentages of oxygen, certain strains of *Clostridium Welchii* develop under less oxygen tension than others, but above a certain amount of free oxygen in the medium, growth never occurs. Repeated artificial cultivation for several generations tends to increase the optimum free oxygen allowed, as shown by the fact that the growth approaches nearer and nearer the surface in agar stab cultures after each additional transfer.

Anaerobiosis is effected by simply sterilizing all media under a thin film of oil (albolin). If the medium has remained in the laboratory more than one month after sterilization, it is boiled for one half hour previous to inoculation or resterilized in the auto-clave at ten pounds pressure for fifteen minutes. Anerobic conditions are obtained with plate cultures by covering the surface of the solidified medium with a thin layer of paraffin at the lowest temperature at which this flows. Media which have been sterilized without albolin permit the growth of *Clostridium Welchii* if inoculated directly after sterilization since this organism develops so rapidly at 37° that free oxygen cannot be absorbed sufficiently in a short time to inhibit development. When grown in this manner its life is extremely short as oxygen is soon absorbed in sufficient amount to destroy it. Although a film of oil renders media suitable for anaerobic growth for a longer time than if sterilized without it, simply boiling the media, or resterilization before using, is the only requisite for the growth and isolation of *Clostridium Welchii*.

2. Food requirements. *Clostridium Welchii* grows upon all of the ordinary culture media, although 1 per cent glucose or maltose stimulates its growth when added to plain meat-extract-peptone agar or broth. To maintain the activity of vegetative forms, transplantations are necessary at least every forty-eight hours. In sugar-free broth, sporulation occurs within two to four days. In broth containing a fermentable substance, all vegetative forms of the organism die within three to five days provided it is present in the vegetative form.

Growth in peptone-free media does not occur. A 1 per cent solution of glucose, maltose or lactose without peptone, a physiological salt solution with 1 per cent glucose, maltose or lactose, a 1 per cent glucose, maltose or lactose, a physiological salt and a 1 per cent gelatin solution fail to show any perceptible growth after two weeks incubation.

The spores of *Clostridium Welchii* can be preserved indefinitely in nearly all media in the absence of glucose, galactose, maltose, lactose, sucrose, dextrin and starch. Media which are vigorously fermented by *Clostridium Welchii* do not permit of its sporulation.

3. Temperature requirements. The optimum temperature for this organism is 37°, growth occuring from 10° to 42°. Below 10° vegetative forms die quickly while spores may remain alive for months if a large quantity are present in the medium.

When vegetative and spore forms of *Clostridium Welchii* are exposed to temperatures below the freezing point, an enormous reduction in numbers is observed. In determining this fact for vegetative forms, pure cultures which had been transferred at least twice into freshly sterilized glucose-liver broth were used. A known number of the organisms from these pure cultures were inoculated into freshly sterilized glucose-liver broth and exposed to temperatures ranging from 13° to 36°F. After each twenty-four hours, the actual bacterial content was determined by the dilution method until the organisms failed to develop in a dilution of one per cubic centimeter. In eight days the bacterial content dropped from 50,000,000 per cubic centimeter to less than one per cubic centimeter with a comparatively uniform rate daily. Of the eight cultures tested, all behaved similarly and produced like results.

With spores grown in peptone-egg media for seven days, a similar reduction occurred, although it took a longer period to cause complete destruction.

Freezing vegetative and spore forms of *Clostridium Welchii* effects a steady death rate which leads to the complete destruction of millions of vegetative forms in the course of seven or eight days, while spores fail to develop after ten to twelve days exposure to this environment.

4. Longevity. The longevity of the vegetative forms has already been discussed. If sugar media are used with meat extract as the base, the life of the vegetative organism is about three days; whereas if liver is used instead of meat extract the organism survives from three to five days. In milk, the organism dies out as a rule in from seventy-two to ninety-six hours. In lactose-liver broth the cultures are in the vegetative stage after nine days incubation. Starch and dextrin-liver-broth preserve the life of the organism for four to six days in some cases, but does not cause sporulation, whereas starch and dextrin broth made from meat extract causes the death of the organism in three to four days. Cultures in sugar media in the incubator die out more rapidly than at room temperature.

Cultures which contain spores may be preserved for many weeks, and in some cases for over a year, provided anaerobic conditions are maintained. The numbers of *Clostridium Welchii* present in any culture and the longevity of the organism is inversely proportional to the length of time necessary for the appearance of "stormy fermentation." A covering of oil prolongs the life of this organism appreciably.

VII. CULTURAL CHARACTERS

1. Agar. a. Gas formation. Gas bubbles appear to some extent in plain agar stab cultures, yet much more abundantly and rapidly in agar containing sugar. In plain agar the presence of gas is delayed from two to ten days, while in sugar agar, fermentation takes place very quickly. Bubbles appear at first along the line of growth, but ultimately permeate the entire medium. At 35° to 37° gas production is most abundant.

On plates of glucose agar, numerous gas bubbles are present after 15 hours growth if the plates are heavily seeded with the organism. From the spaces occupied by the bubbles, as well as upon the surface of the agar, a turbid fluid is pressed out, due to the bacteria present. Deep stab cultures in tubes of glucose agar are accompanied by abundant gas production which results in the fragmentation of the medium and the extrusion of turbid fluid on the surface.

On thickly seeded glucose-liver agar plates, abundant gas production occurs, while on plates containing only a few colonies no gas usually occurs; but occasionally two or three colonies on glucose-liver-agar plates will develop sufficient gas to cause a bubble to appear covering the entire plate under the paraffin. This bubble, if punctured, burns with the blue flame characteristic of hydrogen. Gas formation frequently fails to occur in deep glucose-liver agar stab cultures, when only two or three colonies develop in the lower part of the medium.

Nutrient agar of + 1 reaction with 1 per cent glucose or lactose causes a rapid and luxuriant growth with more abundant and speedy gas formation than occurs on plain nutrient agar without sugar. The odor of plain agar cultures is not putrescent or characteristic. The odor of sugar agar cultures resembles that of sour, stale glue, a condition more pronounced with some strains than others.

b. Colonies. Colonies of *Clostridium Welchii* do not develop in plain meat extract agar unless all the oxygen is first removed and strict anaerobic methods employed in the plating. The more strictly anaerobic the conditions employed the larger the colonies as shown by the fact that the colonies in stab nutrient or sugar agar, are very much larger at the lower part of the needle track. In fact when agar stab cultures are inoculated (after sufficient steaming to dispel the air) growth appears most abundant at the bottom of the stab, ceasing to develop some distance below the surface, according to the degree of the anaerobiosis.

Colonies of *Clostridium Welchii* in plain and sugar agar are opaque white, grayish white, or even of a brownish white color by transmitted light, sometimes with a central darker dot, the opacity of the color depending upon the thickness of the colony. The dark center appears in all media in which the organism sporulates.

Young cultures vary in size from 0.5 to 1 mm. in diameter, but frequently increase to diameters of 2 to 3 mms. or even larger. The colonies in plain agar are very minute pin points with fuzzy edges and with a zone around them resembling a halo. A dark center appears in the colony after ten to twelve

days' incubation. The colonies are much larger in media containing sugar than on plain agar. The size of colonies on glucose-liver-agar varies greatly from minute nearly microscopic colonies on thickly seeded plates to those measuring from 3 to 5 mm. on plates with few colonies.

The colonies in stab cultures, and those deep in the agar on plates, appear as spheres or ovals, generally more or less flattened with irregular contours, the irregularity being due to little feathery projections or prongs from the surface of the colonies. The colonies are very firm, and retain their shape and consistency when touched with a needle.

2. Gelatin. Gas is formed in ordinary nutrient gelatin (reaction + 1) neutral glucose-gelatin and neutral glucose-liver-gelatin. The gas production in these media varies, nutrient gelatin showing the least and glucose-liver gelatin the most abundant production. Growth occurs in all three at 20° and 37°, the glucose-liver cultures developing more rapidly and abundantly than nutrient gelatin cultures.

A slight initial softening is observed in the 20° cultures, due to the peptonization of the gelatin at the top of the stab; later the growth settles downward along the line of puncture, until in some cases the entire medium liquefies.

3. Broth. Growth occurs under strictly anaerobic conditions in sugar-free broth, and in broth containing glucose, sucrose, maltose, lactose, dextrin, starch, liver and inulin. The gas is so abundant in the sugar broth that small bubbles of gas rise to the surface and accumulate to form a "foamy" layer, presenting a "steaming" appearance. At 37° growth takes place in two or three hours, the clear broth first becoming cloudy with abundant sediment at the bottom of the tube. When gas formation starts it goes on with great rapidity for about twenty-four hours in plain broth and still longer in sugar broth. After the fermentation ceases the diffuse cloudiness disappears and the sediment settles in the course of a short time, rendering the cultures clear and transparent. The sediment is white, uniform and flaky and more abundant in sugar broth than in plain broth. If the sediment is disturbed, it floats in a viscid thread or cloud produc-

ing again diffuse cloudiness, which quickly settles upon standing. In a few cases the broth is stringy and very viscuous, yet the sediment settles out leaving the supernatant fluid as in the other cases. After the development is complete, the reaction is decidedly acid. The odor of the cultures is not putrescent but resembles sour, stale glue as in agar and gelatin cultures. Glucose, galactose, plain liver, sucrose, maltose, lactose, starch, dextrin and in a few cultures inulin, lead to a most violent fermentation when added to plain broth. Different strains vary widely in their fermentative ability with glycerol and inulin. An extreme acid production is obtained with all the sugars, the maximum reaction being reached in about forty-eight hours. In peptone water containing coagulated egg-white, physiological salt solution and coagulated egg-white, and an aqueous solution of coagulated egg-white, *Clostridium Welchii* causes the so-called "stormy fermentation" which is characteristic in milk. Mannitol broth, 1 per cent casein solution, plain peptone water and Dunham's solution are not fermented appreciably, less than 10 per cent of gas appearing after three days. In media containing coagulated egg-white a black deposit occurred in the white sediment.

4. Milk. After twenty-four to forty-eight hours' incubation at 37°, milk tubes inoculated with pure cultures of *Clostridium Welchii* showed the so-called "stormy fermentation," coagulation and derangement of the curd with gas bubbles rising to the top of the tube. After an incubation of forty-eight hours the digestion is complete, leaving a yellowish whey. Market milk which has been heated for fifteen minutes at 82° and incubated at 37° shows after twenty-four to seventy-two hours variations of the typical reaction from the typical "stormy fermentation" to a slight digestion of the curd, with little or no gas formation. Where gas formation failed to appear the reaction was due, presumably, to insufficient anaerobiosis of the medium. Frequently a culture from a milk tube showing no gas, when inoculated in other milk tubes freshly sterilized, would cause "stormy fermentation" after incubation. Much acid is produced from this fermentation, its production ceasing only upon the exhaustion of the fermentable materials present. Butyric acid is produced

in large amounts, as is detected by its characteristic odor. Litmus milk turns from blue to red very rapidly after inoculation upon incubation showing the increase of acidity.

5. Potato. *Clostridium Welchii* was grown in broth tubes containing pieces of potato and sterilized under oil. Gas production was rapid and growth was abundant in twenty-four hours. The starch was so vigorously attacked in some cases that the potato was penetrated by the organism. The colonies, which appeared on the potato, were thin, moist and grayish-white on the surface and through the pieces. No spores were demonstrated in this medium, either microscopically or experimentally.

6. Blood serum. Growth is abundant on the smeared surface of blood serum after twenty-four hours incubation at 37°. Digestion of the medium soon takes place, changing the consistency of the coagulated serum and liquefying it completely in from four to six days. Cultures live in this medium for at least six months, spores appearing first on the sixth day of incubation.

The colonies on blood serum appear round, about 7 mm. in diameter, opaque grayish-white with finely granular edges somewhat frayed. A foul odor is given off after several days growth. These facts are based on experiments with 20 strains of *Clostridium Welchii* isolated from market milk and inoculated on Loeffler's blood serum.

VIII. CHEMICAL CHARACTERS

1. Action on carbohydrates. a. Gas production. With all the monosaccharides, disaccharides and polysaccharides used, 100 per cent of gas is produced, the time required for such production being from eighteen to thirty-six hours. In nearly every case little or no gas is formed after forty-eight hours. Inulin is fermented in some cases but mannitol and glycerol are never fermented by any strain of this organism.

b. Acid production. The amount of acid produced by the growth of *Clostridium Welchii* in sugar media ranges from less than 1 to 12 per cent as determined by titrating against $\frac{N}{10}$ alkali,

using phenolphthalein as indicator. The most abundant acids are lactic, acetic and butyric. The acidity in all cases attains its maximum between thirty-six and forty-eight hours incubation at 37°, after which there is a slight drop to a value which remains constant indefinitely, regardless of the longevity of the organism.

2. Action on proteins. *Clostridium Welchii* is characteristically a fermentative organism and attacks proteins very slightly, and then only in the absence of fermentable carbohydrates. If sugar is present in a medium containing protein, the organism acts upon it readily, leaving the protein unattacked. Egg-meat mixture is not attacked appreciably, but produces a disagreeable odor upon standing. The egg-meat mixture used is prepared as follows:

A. One-half pound of lean chopped beef is placed in 250 cc. of water, neutralized with sodium carbonate and heated for thirty minutes in the Arnold sterilizer with occasional stirrings. This is then set away in a cold place for several hours after which the fatty scum is removed.

B. The whites of three eggs are placed in 250 cc. of water, neutralized and heated with occasional stirring in the Arnold sterilizer for thirty minutes to coagulate the albumen.

A and B are mixed and 2.5 grams of 0.5 per cent calcium carbonate (powdered) are added. The flask is plugged and sterilized for thirty minutes at 112°.

When this mixture is inoculated with *Clostridium Welchii* there appears just the slightest fermentation due to the small amount of sugar present in the meat and egg.

Growth occurs in a 1 per cent casein solution, but the casein is unattacked by the different strains isolated from milk and feces. Peptone water alone and peptone in physiological salt solution remain unattacked. In liver broth, the glycogen is acted upon very readily with vigorous gas fermentation, whereas the protein is slightly, if at all affected. On albuminous matter alone, however, there appears some proteolytic activity which, when a little sugar is present, is increased enormously.

No evidence of fermentation occurred in cultures of *Clostridium Welchii* grown in peptone water, egg-white and egg-yolk solutions,

a 1 per cent casein solution, and egg-meat mixture incubated for one week at 37.5. In the case of peptone egg-white 100 per cent of gas forms after a week's incubation. After two weeks' growth spores are observed in all the media except casein.

While there appeared to be a slight decrease in the bulk of the proteins in some instances, as well as disagreeable odors, the transformation never assumed the character of real putrefaction. The unpleasant odor is presumably due to the presence of butyric and closely allied acids and not to any product of decomposition of proteins.

By putrefaction is meant the ability to decompose native proteins with the formation or liberation of foul-smelling products, mercaptan, aromatic oxy-acids, etc. Rettger, 1908, in his "Studies on Putrefaction" claims that not all obligate anaerobes have the property of producing putrefaction and has divided the strict anaerobes into four classes, in so far as their bio-chemical characters are concerned, as follows.

First, those that produce very little or no putrefactive changes or fermentation with evolution of gas; second, those that have a strong putrefactive action on native proteins but fail in fermentative properties; third, those which are primarily fermentative organisms and whose putrefactive functions are very slight or perhaps absent; and fourth, those which have very marked putrefactive and fermentative properties. Herter, 1907, ascribes marked putrefactive properties to *Clostridium Welchii* and regards it as being largely responsible for certain intestinal disturbances although he uses the term putrefaction in a much broader sense. Passini, 1905, also asserts that the bacillus of gaseous phlegmon produces an abundance of indol when grown in pure culture on blood serum. This is contrary to my observations on indol production. Tissier and Martelly, 1902, claimed that *C. perfringens* isolated from putrefying meat and resembling *Clostridium Welchii* had a decided putrefying action on blood fibrin although the action was slow. Notwithstanding these views, using the foregoing classification of Rettger's (1908), *Clostridium Welchii* from my observations on the action of sugar-free native proteins would fall in his third class of anaerobes, namely "primary

fermentative and whose putrefactive functions are very slight or absent."

3. Enzymes. Enzyme action is shown in the liquefaction of gelatin and blood serum and the peptonization of milk casein. Diastatic enzymes may be detected by growing cultures of *Clostridium Welchii* in starch solution when the starch is converted into sugars.

IX. CLASSIFICATION

1. Historical. The success that has attended previous investigators in the use of certain carbohydrates and other fermentable substances as a means of differentiating between the different members of a group suggested the use of these substances as a means of distinguishing members of the *Clostridium Welchii* group.

Little has been accomplished in the separation of the members of this group due presumably to the fact that the differences are so small and that it has been impossible to obtain uniform conditions.

Fraenkel, 1902, and Klein, 1900–1902, recognized only one variety of the organisms isolated by them. Schattenfroh and Grassberger, 1900, and Passini, 1905, differentiated two species, virulent and non-virulent. Hitschmann and Lindenthal, 1899, were unable, either from the behavior in the animal body or in cultures, to differentiate varieties. They found tests of pathogenicity especially unreliable as a means of differentiation. Passini, 1904, Werner, 1905, and Rocchi, 1911, by means of serological reactions attempted to distinguish subvarieties but failed. Herter, 1907, was also unsuccessful in his attempt to apply such factors as sporulation, pathogenicity, hemolytic properties, indol production and rapidity of gas formation in man and animals.

Jackson, 1912, described two types, one motile, neutral reaction with no gas formation in lactose bile and 56 per cent gas production in mannitol broth; the other non-motile, 92 per cent gas production in lactose bile and 10 per cent gas production in mannitol broth.

Simonds, 1915, based his classification upon a study of 30 strains according to their fermentative ability in inulin and glycerol broths and their ability to sporulate in neutral media containing these substances. He was able to divide the group into four sub-groups, but he concludes that the source of the culture is no indication of the sub-group to which it belongs although all strains isolated from cow feces and milk fall into the same sub-group.

2. Experimental. The results obtained by this study of over 100 strains isolated from different sources show two possibilities for the classification of *Clostridium Welchii*, namely, (1) the fermentative reactions in glycerol and inulin broth with sporulation in these media and (2) the frequency curves derived from the fermentation of the carbohydrates, glucose, galactose, lactose, maltose, sucrose, starch, dextrin and liver broth. Classification of the *Clostridium Welchii* group could not be effected by the use of such factors as, longevity in different sugars and nutrient broth, sporulation in media of different reactions ranging from 10 per cent acid to 3 per cent alkaline media, thermal death points of vegetative and spore forms, fermentation with acid production in liver media containing the above named carbohydrates, pathogenicity and its relation to virulent and avirulent types, fermentative reactions in protein-free media, liquefaction of gelatin and blood serum, rapidity of gas formation in liver media and animals, motility and the retention of the gram stain. Where variation occurred in these respects there was no characteristic sufficiently stable to serve as a basis for classification. In most of the tests applied, the results were constant throughout and showed the same characteristic with little or no variation.

The pathological study of the organism, as has been discussed in a previous section, reveals interesting and enlightening facts yet they fail to show any characteristic classification. In all cultures isolated from human feces very rapid pathogenic results follow, while from the cow, varying results are obtained. For the most part, however, the bacillus isolated from cow feces fails to set up a characteristic fatal necrosis of the subcutaneous tissue.

a. Classification of *Clostridium Welchii* by fermentative reactions in glycerol and inulin broths. The fermentative reactions of glycerol and inulin broth with sporulation in these neutral media, showed that the members of this group were divided into four subgroups. Fifty-six strains of *Clostridium Welchii* isolated from the following sources: Milk (pasteurized and raw) 42; human stools (normal) 5; cow feces 9; were subjected to this test. All conditions were standardized as far as possible. Pure cultures were obtained through sufficient plating of these organisms and were grown in glucose-liver broth tubes and incubated for another twenty-four hours, after which two drops of these cultures were inoculated into glycerol and inulin. The broths were prepared by rendering neutral to phenolphthalein a 1 per cent solution of peptone and inulin and a 1 per cent solution of peptone and 6 per cent of glycerol. After a forty-eight hour incubation at 37°, the tubes were removed from the incubator and titrated against $\frac{N}{10}$ NaOH with phenolphthalein as an indicator and the acidity recorded. Gas fermentation and sporulation was also determined for all the strains.

Tables 2 and 3 give the results of this study of 56 strains, the four principal subgroups being characterized as follows:

Subgroup 1. Cultures not producing acid or gas from either inulin or glycerol and forming spores in both inulin and glycerol broths. Pathogenicity variable.

Subgroup II. Producing acid with or without gas in inulin but not from glycerol and forming spores in glycerol broth. Pathogenic for guinea-pigs in most cases.

Subgroup III. Producing acid without gas from glycerol but not from inulin and forming spores in inulin broth. Pathogenicity variable.

Subgroup IV. Producing acid from both glycerol and inulin with or without gas production with no formation of spores. Pathogenic in most cases.

The source of the culture gives no indication as to the subgroup to which it belongs as shown by the grouping of the above strains.

TABLE 1

Study of 56 strains from milk, cow feces, and human feces, with regard to acid production and gas, divided into 4 sub-groups according to fermentation of inulin and glycerol

SOURCE	NUMBER	GLUCOSE		GALACTOSE		MALTOSE		LACTOSE		SUCROSE		DEXTRIN		STARCH		INULIN		GLYCEROL		PEPTONE H_2O
		Acid	G	Acid	G	Acid	G	Acid	G	Acid	G	Acid	G	Acid	G	Acid	G	Acid	G	Acid G
Subgroup I																				
Milk	7	2.0+		1.2+		1.2+		1.9+		2.6+		1.9+		2.55+		0	0	0	0	0.4−
	18	2.45+		1.7+		1.25+		1.9+		2.7+		2.25+		2.4+		0	0	0	0	0.4−
	20	2.0+		1.2+		1.9+		2.0+		2.6+		3.05+		3.3+		0	0	0	0	0.4−
	21	2.35+		1.15+		0.85+		1.35+		2.65+		2.75+		2.45+		0	0	0	0	0.35−
	24	2.3+		1.15+		1.2+		1.4+		2.7+		1.9+		2.3+		0	0	0	0	0.4−
	26	1.95+		1.15+		1.3+		1.4+		2.05+		2.4+		2.5+		0	0	0	0	0.4−
	1	1.8+		1.3+		1.35+		1.6+		2.55+		3.1+		2.5+		0	0	0	0	0.4−
	6	2.35+		1.25+		1.65+		0.55+		2.95+		2.1+		1.85+		0	0	0	0	0.35−
	C	2.3+		1.2+		1.55+		1.55+		2.6+		3.00+		2.5+		0	0	0	0	0.35−
	36	2.2+		1.0+		1.6+		1.6+		2.6+		2.6+		2.6+		0	0	0	0	0.5−
	T	2.8+		1.85+		1.65+		1.85+		2.65+		2.4+		2.4+		0	0	0	0	0.35−
	R	2.3+		1.5+		1.1+		1.4+		1.0+		2.7+		2.7+		0	0	0	0	0.4−
Cow feces	5	1.9+		0+		1.1+		2.05+		2.6+		3.00+		2.5+		0	0	0	0	0.45−
	7	2.1+		0+		1.8+		0.75+		2.6+		0.6+		3.00+		0	0	0	0	0.4−
	11	2.15+		0+		1.1+		2.0+		2.6+		0.55+		3.00+		0	0	0	0	0.5−
	13	2.3+		0.65+		1.7+		2.1+		2.6+		0.5+		1.6+		0	0	0	0	0.4−
	12	1.85+		0.5+		2.0+		2.0+		2.5+		0.25+		2.6+		0	0	0	0	0.5−

Subgroup II

Milk	2	2.1+	0.9+	1.9+	1.3+	2.9+	3.1+	2.45+	1.2	30	0	0	0.4−
	P	2.55+	1.25+	1.85+	1.7+	2.6+	1.65+	1.35+	0.8	40	0	0	0.35−
	25	2.45+	1.2+	1.7+	1.7+	2.6+	1.7+	1.5+	1.4	30	0	0	0.2−
	45	2.05+	1.2+	1.2+	1.4+	2.7+	2.35+	1.9+	0.6	40	0	0	0.3−
	Y	2.25+	1.35+	0.55+	0.65+	2.7+	2.4+	2.1+	1.2	30	0	0	0.4−
	K	2.55+	1.55+	0.8+	1.65+	3.1+	3.15+	2.95+	0.85	30	0	0	0.35−
Cow feces	4	2.0+	1.2+	1.1+	2.05+	2.6+	2.5+	1.8+	0.8	40	0	0	0.5−
	8	1.9+	0+	1.0+	2.0+	2.6+	2.5+	+	1.05	0	0	0	0.6−
	17	2.4+	0+	1.0+	2.0+	2.45+	+	+	1.05	0	0	0	0.5−
Human feces	6	2.55+	0.3+	1.7+	1.2+	2.8+	2.9+	3.15+	1.5	0	0	0	0.4−
	5	2.4+	1.6+	1.8+	1.5+	2.8+	2.9+	3.15+	1.1	0	0	0	0.3−

Subgroup III

Milk	3	2.5+	1.6+	2.05+	1.7+	3.0+	2.65+	2.2+	0	0	0.50	0	0.3	0
	4	2.3+	1.55+	1.6+	1.7+	3.1+	2.85+	2.75+	0	0	0.30	0	0.4	0
	9	2.35+	1.2+	1.4+	1.8+	2.5+	1.9+	2.35+	0	0	0.20	0	0.4	0
	11	1.9+	0.9+	1.4+	1.4+	2.5+	1.6+	2.35+	0	0	0.30	0	0.4	0
	12	2.5+	1.3+	1.1+	1.55+	2.75+	2.6+	2.3+	0	0	0.55	0	0.45	0
	13	2.2+	1.5+	1.35+	1.95+	3.05+	2.75+	2.45+	0	0	0.45	0	0.35	0
	17	2.6+	0.85+	1.7+	1.50+	3.00+	1.75+	1.7+	0	0	0.30	0	0.3	0
	22	2.55+	1.9+	2.1+	2.0+	1.4+	2.25+	2.55+	0	0	0.80	0	0.3	0
	J	1.95+	1.3+	0.75+	1.4+	2.55+	2.65+	2.2+	0	0	0.35	0	0.4	0
	N	2.95+	1.8+	1.35+	2.0+	1.1+	2.8+	2.45+	0	0	1.15	0	0.3	0
	U	2.25+	1.25+	1.45+	1.4+	2.25+	2.3+	2.2+	0	0	0.35	0	0.3	0
	40	2.0+	0.8+	1.4+	1.4+	2.15+	2.4+	1.9+	0	0	0.20	0	0.5	0
	42	1.95+	1.25+	1.75+	0.7+	2.7+	2.2+	1.85+	0	0	0.30	0	0.5	0
	47	2.45+	1.45+	1.95+	1.95+	1.15+	2.05+	2.5+	0	0	1.15	0	0.35	0
	27	2.8+	1.5+	0.95+	1.45+	1.5+	2.05+	3.1+	0	0	1.15	0	0.35	0
Cow feces	10	1.85+	0+	0.8+	2.0+	2.5+	2.5+	2.6+	0	0	1.15	0	0.50	0

TABLE 2—*Continued*

SOURCE	NUMBER	GLUCOSE	GALACTOSE	MALTOSE	LACTOSE	SUCROSE	DEXTRIN	STARCH	INULIN		GLYCEROL		PEPTONE H_2O	
		Acid G	Acid G	Acid G	Acid G	Acid G	Acid G	Acid G	Acid G		Acid G		Acid G	
Subgroup IV I														
Milk	7	2.55+	1.6+	1.05+	1.4+	3.3+	2.55+	2.95+	0.8	30	0.40	0	0.4	0
	8	2.45+	1.55+	1.7+	1.65+	2.8+	2.1+	2.55+	0.8	30	0.40	0	0.4	0
	E	1.85+	1.65+	0.95+	0.65+	2.5+	2.55+	2.25+	0.5	0	0.55	0	0.35	0
	Q	3.05+	1.25+	0.55+	+	1.1+	2.35+	2.35+	1.45	25	1.15	0	0.35	0
	S	2.05+	1.5+	1.65+	1.95+	2.6+	1.7+	1.6+	0.65	50	0.80	0	0.35	0
	V	2.00+	1.25+	1.6+	1.1+	2.3+	2.5+	1.8+	0.6	0	0.35	0	0.4	0
	22	2.6+	1.45+	1.1+	1.05+	3.0+	2.5+	2.5+	1.05	30	1.65	0	0.3	0
	23	2.1+	1.3+	1.15+	0.7+	2.8+	2.7+	1.8+	0.6	25	0.65	0	0.4	0
	31	2.7+	0.5+	1.45+	1.45+	3.25+	2.5+	2.85+	1.4	20	0.6	0	0.45	0
Human feces	4	2.3+	1.65+	1.2+	1.5+	2.75+	2.5+	2.3+	0.7	0	0.8	25	0.4	0
	7	2.0+	1.25+	0.55+	0.55+	2.55+	2.55+	3.0+	0.7	0	0.3	0	0.45	0
	10	1.95+	0.7+	1.75+	0.55+	2.5+	0.75+	3.0+	1.05	0	0.4	0	0.25	0

TABLE 3

Summary of source of principal subgroups

SUBGROUP	MILK	COW	HUMAN	TOTAL
I	12	5	0	17
II	6	3	2	11
III	15	1	0	16
IV	9	0	3	12
	42	9	5	56

b. Frequency curves. Using the results obtained from a study of the 56 strains used in this classification, frequency curves were plotted for the carbohydrates, glucose, galactose, maltose, lactose, sucrose, starch, inulin, glycerol and dextrin and liver.

The curves show an entirely irregular variability without any particular significance.

X. THERMAL DEATH POINT

1. Method. The thermal death point of strains of *Clostridium Welchii* was determined both in the vegetative and spore stage according to the procedure in Standard Methods for Water Analysis, 1912, except that five loopfuls of the culture were used instead of three in order to overcome the influence of the film of oil and insure inoculation of the organism. Cultures were plated at least twice to insure their purity and if on microscopical examination any contamination was present, plating was repeated until a pure culture was obtained. The pure cultures were grown in glucose-liver broth for twenty-four hours and then transferred to fresh glucose-liver broth and grown for another twenty-four hours.

The sources from which *Clostridium Welchii* was isolated to study thermal death point were market milk, human feces, cow feces, and horse feces. In the case of the cultures of market milk, some had been subjected to artificial cultivation for four months, others for three months, and still others for two weeks before testing their thermal death point while the cultures from

the fecal sources were tested as soon as possible after they were isolated in pure culture. For the determination of the thermal death point, tests were made on 75 cultures from market milk, 54 cultures from human feces, 18 from cow feces and 18 from horse feces. The thermal death point of the spores in cow and horse feces was not determined.

2. Thermal death point of vegetative forms. Tubes containing 10 cc. of glucose-liver broth were inoculated with two-tenths cc. of the pure cultures and grown for eight, twenty-four and forty-eight hours at 37.5°. Cultures were also grown in sterile milk. The best and most consistent results were obtained by growing at 37.5° for eight hours. A double water-bath was used to keep the temperature uniform. This bath was heated to the desired temperature and a sufficient supply of tubes each containing 10 cc. of sterile glucose-liver broth was placed in the inner bath. The temperature of the bath was recorded in a control tube by a thermometer placed in the broth which was not allowed to vary more than half a degree in either direction. The water in the bath was agitated by means of a stirring rod throughout the heating. After fifteen minutes exposure at the desired temperature, the broth was inoculated with five loopfuls of the eight-hour broth culture to be tested by simply removing the cotton plug, without removing the tube from the bath. No attempt was made to determine the numbers per cubic centimeter before and after the heat exposure, either of the vegetative or the spore forms. The amount was kept as uniform as possible using the same size loop and uniform 1 cc. pipettes. The inoculated tubes were exposed for fifteen minutes at this temperature and were then transferred at once to a vessel of cold water in order to cool them quickly and prevent further action of the heat upon the bacteria. When cold they were placed in the incubator at 37.5° and kept under observation long enough to ascertain whether growth occurred. *Clostridium Welchii* in the vegetative stage showed evidence of growth in twelve to forty-eight hours if the heat had not killed the organism. Duplicate tubes were inoculated to check the determination and in practically all cases the same thermal death point was obtained.

In no case was there more than a degree difference in duplicate samples. Control tubes were always run at 37° and 80° for fifteen minutes to determine whether the organism was alive before heating and to ascertain the presence of spores. All cultures used showed the characteristic "stormy fermentation" of milk previous to the test. The temperatures used to determine the thermal death point of vegetative forms were 56°, 57°, 58°, 59°, 60°, 61°, 62°, 63° and 80°.

3. Thermal death point of spores. The determination of the thermal death point of *Clostridium Welchii* in the spore stage was determined in a manner similar to that described above using the same pure cultures grown for eight hours by inoculating 0.5 cc. of the "steaming" culture into a neutral medium of peptone-egg and growing at 37.5° for one week. At the end of the seven days' incubation, the resistance of the spores to high temperatures was determined. The cultures to be tested were subjected to temperatures from 85° to 101°, raising the temperature one degree at a time. All the spore cultures survived 86° and some survived 100° for fifteen minutes and thirty minutes. The tubes containing the inoculated material were cooled immediately after the fifteen minute exposure at the desired temperature and incubated at 37° until definite results were obtained. In the majority of cases twenty-four hours' incubation was sufficient for the organism to germinate, but in some cases germination did not occur until after four or five days in the incubator. When doubtful results were obtained, 0.5 cc. of the culture containing spores was inoculated instead of the five loopfuls to make sure that there were sufficient spores present. The use of 0.5 cc. was however resorted to only in exceptional cases, for where negative results were obtained with five loopfuls, the same result was found with the larger quantity.

4. Discussion of results. a. Vegetative forms. The thermal death point of vegetative forms of *Clostridium Welchii* varies according to the source from which the cultures are isolated and the duration and conditions of growth previous to the test. Cultures isolated from market milk survive 56° and die at 61°, although cultures immediately after isolation die at 59°. The

thermal death point of the vegetative forms in human feces is from 59° to 61°. In no case does a vegetative culture survive 63°, provided no spores are present. This is the temperature of pasteurization. Using the data obtained from this work, frequency curves showed no indication of more than one type in this group.

b. Spores. In the case of spores of *Clostridium Welchii*, there seem to be two thermal death points, namely, either the cultures resist 100° for fifteen minutes and longer or they die below 90°. Some of the cultures survive 100° for thirty and forty minutes. Two exceptions arise in the cases of number 11 and number 13 which fail to survive above 95°. Repeated tests confirmed the failure of these strains to survive boiling. Spores from human feces do not survive boiling in any case. Frequency curves show conclusively that the thermal death points of spores fall into two distinct groups.

c. Relationship between vegetative forms and spores. An inspection of the charts shows there is no correlation between the thermal death points of the spores and the vegetative forms for the thermal death point of vegetative forms may range from 56° to 60°, while spore forms of corresponding cultures resist 100°. Moreover, spores dying at 86° to 88° may resist 58° and 59° in the vegetative stage. It is impossible to predict the thermal death point of the spore culture provided the thermal death point of the vegetative culture for the same strain is known or vice versa.

d. Classification of sugar fermentation as compared to thermal death points. The classification of the members of the *Clostridium Welchii* group into four subgroups on a basis of the fermentative reactions in glycerol and inulin, as shown in a preceding section, bears no relationship to the two distinct groups based on the thermal death points of spores. Each of the four subgroups in the classification according to fermentation contains members of both groups of thermal death points. The same is true of the vegetative forms, each group containing cultures whose resistance to heat varies widely.

e. Relation of the time since isolation to the thermal death point. Table 4 shows the effect of prolonged artificial culti-

TABLE 4

Thermal death points

NUMBER ORIGINAL	TEMPERATURE BEFORE TESTING																						
	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	55	56	57	58	59	60	61	62
	Spores																	Vegetative							
Cultures isolated from milk and kept four months before testing																									
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
3	+	+	+	+	+	−	−	−	−	−	−					−	−	+	+	+	+	−	−	−	−
4	+	+	+	−	−	−	−	−	−	−	−					−	−	+	+	+	+	−	−	−	−
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
7	+	+	+	+	−	−	−	−	−							−	−	+	+	+	+	−	−	−	−
8	+	+	+	−	−	−	−	−	−							−	−	+	+	+	+	−	−	−	−
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
11	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	+	+	+	+	−	−	−
12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−	−	−
13	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−
17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	−	−
18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	−	−
19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
14	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	+	−	−	−	
Cultures isolated from milk and kept three months before testing																									
A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
B	+	+	−	−	−	−	−									−	−	+	+	+	+	−	−	−	−
C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
E	+	+	+	−	−	−	−									−	−	+	+	+	−	−	−	−	•
F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−	−	−	
G	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−	−	−	
H	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−	−	−	
I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	
J	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−		
K	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−		
L	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−	−	−	
M	+	+	+	+	+	+	−	−	−	−						−	−	+	+	+	−	−	−	−	
N	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−	−	−	
O	+	+	+	+	−	−	−	−	−							−	−	+	+	+	+	−	−	−	
P	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	
Q	+	+	+	+	−	−	−	−	−							−	−	+	+	+	+	−	−	−	
R	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	
S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	
T	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−	−	
U	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−	−	

TABLE 4—*Continued*

NUMBER ORIGINAL	TEMPERATURE BEFORE TESTING																							
	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	55	56	57	58	59	60	61	62
	Spores																	Vegetative							
V	+	+	+	−	−	−	−									−	−	+	+	+	+	−	−	−	
W	+	+	+	−	−	−	−									−	−	+	+	+	+	−	−	−	
X	+	+	−	−	−	−	−									−	−	+	+	+	+	−	−	−	
Y	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	−	
Cultures isolated from milk and kept one and a half months before testing																									
45	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	
46	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	
47	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	
48	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	
49	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	
50	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	
Cultures isolated from milk and kept two weeks before testing																									
1 20	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−		
1 21	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−		
3 22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−		
3 23	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−		
3 24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−		
3 25	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−		
6 26	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−		
6 27	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−		
6 28	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−		
6 29	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−		
6 30	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−		
6 31	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−		
10 32	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−		
10 33	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−		
10 34	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−		
10 35	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−		
11 36	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−		
11 37	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−		
14 38	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−		
14 39	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	
20 40	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−		
20 41	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−		
20 42	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−		
20 43	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−		
20 44	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−		

vation on the thermal death point of vegetative forms of *Clostridium Welchii*. Of 20 strains isolated from market milk and tested after four months' cultivation on artificial media, all were negative at 61°, two survived 60°, nine survived 59° and all survived 58°. Of 24 strains from market milk grown for three months all failed to survive 60°, two survived 59°, 14 survived 58° and all survived 57°. These cultures were kept in the spore form in peptone egg for a month and a half before testing, whereas the first 20 strains were transferred daily or every forty-eight hours to fresh sugar media during the four months. Twenty-five freshly isolated strains grown for two weeks gave the following results:— all survived 56°, 18 survived 57° and eight survived 58°. All cultures after four months survived 58°, after three months 57°, while the corresponding value for freshly isolated strains was only 56°.

Of the spore forms seven out of 20 (35 per cent) failed to survive boiling after four months' growth, eight out of 24 (33⅓ per cent) failed to survive boiling after three months' growth and 15 out of 25 (60 per cent) after two weeks' growth.

f. Relation of age of cultures and kind of medium to thermal death point. Tables 5 and 6 show the greater resistance to heat of cultures from milk and feces when grown in glucose-liver broth for more than eight hours before testing. In 13 out of 20 cultures from market milk, growth for twenty-four hours increases the thermal death point 1°. In the other cases no difference was observed under identical conditions. In 19 cultures from human feces, growth for forty-eight hours increased the thermal death point 1° in every case.

Table 5 gives also the results of 19 cultures grown in sterile milk for sixty-six hours in comparison to the growth in glucose-liver broth and shows variations in resistance to heat. Seven of the cultures had the same thermal death point as in the eight-hour glucose-liver broth cultures, six cultures resisted 1°C. higher while the remaining six survived a rise of 2°C. above the eight-hour cultures Probably cultures isolated from feces and grown in sterile milk for eight hours approximate the same resistance to heat as the milk cultures themselves.

g. Thermal death point to cultures isolated from feces and milk. Tables 7 and 8 show the variation in thermal death point of cultures freshly isolated from milk and feces when subjected to the same conditions. Of 25 cultures from market milk all survived 56°, 18 survived 57°, and eight survived 58°;

TABLE 5

Thermal death points. Cultures freshly isolated from human feces and grown for eight hours and twenty-four hours in glucose-liver broth and sixty-six hours in sterile milk

NUMBER	EIGHT HOURS—GLUCOSE-LIVER BROTH							TWENTY-FOUR HOURS—GLUCOSE-LIVER BROTH							SIXTY-SIX HOURS—STERILE MILK						
	Temperature							Temperature							Temperature						
	57	58	59	60	61	62	63	80	57	58	59	60	61	62	63	80	57	58	59	60	61	62	63	80
	Vegetative								Vegetative								Vegetative							
1	+	+	+	−	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	−	−	−	−	−
2	+	+	+	−	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	+	−	−	−
6	+	+	+	−	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	+	−	−	−
8	+	+	+	−	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	−	−	−	−
10	+	+	+	−	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	−	−	−	−
13	+	+	+	−	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	+	−	−	−
16	+	+	+	−	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	+	−	−	−
19	+	+	+	−	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	+	−	−	−
4	+	+	+	+	−	−	−	−	+	+	+	+	+	−	−	−	+	+	+	+	−	−	−	−
5	+	+	+	+	−	−	−	−	+	+	+	+	+	−	−	−	+	+	+	+	−	−	−	−
12	+	+	+	+	−	−	−	−	+	+	+	+	+	−	−	−	+	+	+	+	+	−	−	−
14	+	+	+	+	−	−	−	−	+	+	+	+	+	−	−	−	+	+	+	+	+	+	−	−
15	+	+	+	+	−	−	−	−	+	+	+	+	+	−	−	−	+	+	+	+	+	−	−	−
17	+	+	+	+	−	−	−	−	+	+	+	+	+	−	−	−	+	+	+	+	−	−	−	−
3	+	+	+	+	+	−	−	−	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−
7	+	+	+	+	+	−	−	−	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−
9	+	+	+	+	+	−	−	−	+	+	+	+	+	+	−	−	+	+	+	+	+	−	−	−
11	+	+	+	+	+	−	−	−	+	+	+	+	+	+	−	−	+	+	+	+	+	−	−	−
18	+	+	+	+	+	−	−	−	+	+	+	+	+	+	−	−	+	+	+	+	+	−	−	−

Spores did not survive 100°C. in any strain.

of 55 cultures from human feces, all survived 59°, 21 survived 60°, and five survived 61°; of 18 cultures from cow feces, all survived 59°, 16 survived 60°, 13 survived 61°, and five survived 62°; and of 18 cultures from horse feces, all survived 57°, 11 survived 58°, ten survived 59° and three survived 60°.

TABLE 6

Thermal death points. Showing greater resistance to heat after longer periods of growth and uniformity of subcultures from same colony. Full line indicates twenty-fourth hour culture. Dotted line indicates eight hour culture. I_B and I_A = subcultures from original sample. 1, 2, 3, 4 = single colonies from subcultures I_B and I_A

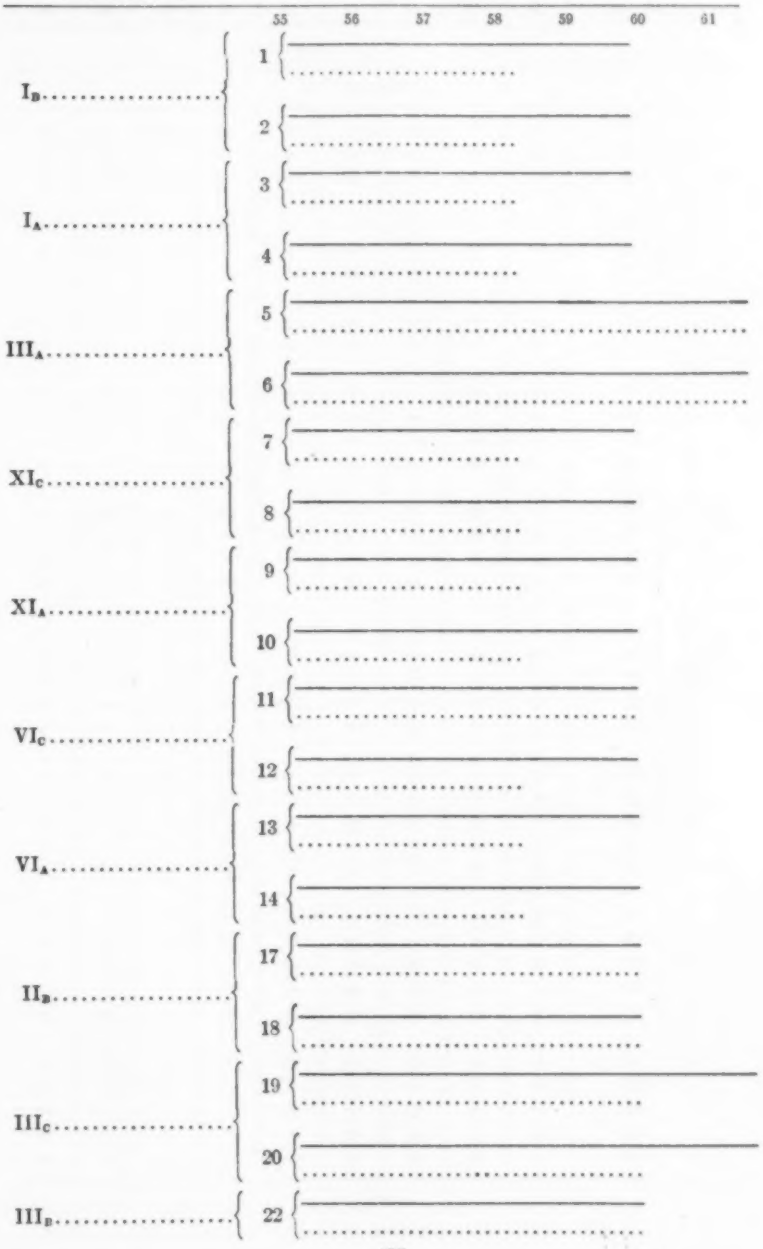

TABLE 7

Thermal death points. Cultures freshly isolated from feces

NUMBER ORIGINAL		TEMPERATURE								
		55	56	57	58	59	60	61	62	63
		Vegetative								
Human										
N_s	1	+	+	+	+	+	−	−	−	−
	2	+	+	+	+	+	−	−	−	−
	3	+	+	+	+	+	−	−	−	−
N_s	4	+	+	+	+	+	−	−	−	−
	5	+	+	+	+	+	−	−	−	−
	6	+	+	+	+	+	−	−	−	−
E_{II}	7	+	+	+	+	+	−	−	−	−
	8	+	+	+	+	+	+	−	−	−
G_s	9	+	+	+	+	+	−	−	−	−
	10	+	+	+	+	+	−	−	−	−
	11	+	+	+	+	+	−	−	−	−
	12	+	+	+	+	+	−	−	−	−
I	13	+	+	+	+	+	−	−	−	−
	14	+	+	+	+	+	−	−	−	−
	15	+	+	+	+	+	−	−	−	−
	16	+	+	+	+	+	−	−	−	−
III	17	+	+	+	+	+	+	−	−	−
	18	+	+	+	+	+	−	−	−	−
	19	+	+	+	+	+	+	−	−	−
	20	+	+	+	+	+	−	−	−	−
	21	+	+	+	+	+	−	−	−	−
IV	22	+	+	+	+	+	−	−	−	−
	23	+	+	+	+	+	+	−	−	−
	24	+	+	+	+	+	+	−	−	−
	25	+	+	+	+	+	−	−	−	−
	26	+	+	+	+	+	+	−	−	−
V	27	+	+	+	+	+	−	−	−	−
	28	+	+	+	+	+	+	−	−	−
	29	+	+	+	+	+	−	−	−	−
	30	+	+	+	+	+	−	−	−	−
	31	+	+	+	+	+	+	−	−	−
	32	+	+	+	+	+	+	−	−	−
	33	+	+	+	+	+	−	−	−	−
	34	+	+	+	+	+	−	−	−	−
	35	+	+	+	+	+	−	−	−	−
	36	+	+	+	+	+	+	−	−	−

TABLE 7—*Continued*

NUMBER ORIGINAL	TEMPERATURE								
	55	56	57	58	59	60	61	62	63
	Vegetative								
Horse									
1 (1)	+	+	+	−	−	−	−	−	−
1 (2)	+	+	+	−	−	−	−	−	−
2 (3)	+	+	+	−	−	−	−	−	−
2 (4)	+	+	+	+	+	−	−	−	−
3 (5)	+	+	+	+	+	+	−	−	−
3 (6)	+	+	+	+	−	−	−	−	−
5 (7)	+	+	+	+	+	−	−	−	−
5 (8)	+	+	+	+	+	−	−	−	−
5 (9)	+	+	+	−	−	−	−	−	−
10 (10)	+	+	+	−	−	−	−	−	−
10 (11)	+	+	+	−	−	−	−	−	−
12	+	+	+	−	−	−	−	−	−
12 (13)	+	+	+	+	+	−	−	−	−
12 (14)	+	+	+	+	+	−	−	−	−
13 (15)	+	+	+	+	+	+	−	−	−
13 (16)	+	+	+	+	+	+	−	−	−
14 (17)	+	+	+	+	+	−	−	−	−
14 (18)	+	+	+	+	+	−	−	−	−
Cow									
1	+	+	+	+	+	+	+	−	−
2	+	+	+	+	+	+	+	+	−
3	+	+	+	+	+	+	+	−	−
4	+	+	+	+	+	+	−	−	−
5	+	+	+	+	+	+	+	−	−
6	+	+	+	+	+	+	+	−	−
7	+	+	+	+	+	+	+	+	−
8	+	+	+	+	+	+	+	−	−
9	+	+	+	+	+	+	+	−	−
10	+	+	+	+	+	−	−	−	−
11	+	+	+	+	+	+	+	+	−
12	+	+	+	+	+	+	+	+	−
13	+	+	+	+	+	+	−	−	−
14	+	+	+	+	+	+	+	−	−
15	+	+	+	+	+	+	+	+	−
16	+	+	+	+	+	+	+	−	−
17	+	+	+	+	+	−	−	−	−
18	+	+	+	+	+	+	−	−	−

Spores from human feces did not survive 100°C.
Thermal death points of spores not determined for these cultures.

Vegetative cultures of *Clostridium Welchii* isolated from the feces of man, cow and horse were more resistant than those isolated from milk. In all cases, cultures from feces survived 59° for fifteen minutes while cultures from milk survived only 56°. Cultures from cow feces were the most resistant, 28 per cent surviving 62°C. Pasteurization at 63°, however, killed all the vegetative forms of *Clostridium Welchii.*

TABLE 8

Thermal death points. Table showing numbers of cultures from different sources surviving temperatures vs. 56° to 63°C.; numbers surviving boiling in spore stage

SOURCE	TIME SINCE ISOLATION	TOTAL NUMBER CULTURES	TEMPERATURE °C.								SPORE
			56	57	58	59	60	61	62	63	
			Vegetative eight hours growth								
Milk	4 months	20	20	20	20	9	2	0	0	0	13
	3 months	24	24	24	14	2	0	0	0	0	16
	1½ months	6	6	6	5	0	0	0	0	0	3
	2 weeks	25	25	18	8	0	0	0	0	0	10
Human	2 weeks	19	19	19	19	19	11	5	0	0	0
	Freshly isolated	36	36	36	36	36	10	0	0	0	0
Cow	Freshly isolated	18	18	18	18	18	16	13	5	0	
Horse	Freshly isolated	18	18	18	11	10	3	0	0	0	
Combined		166	166	159	131	94	42	18	5	0	

Conclusions. The thermal death point of vegetative forms of *Clostridium Welchii* varies from 56° to 63° for fifteen minutes and is not significant in the differentiation of the members of the *Clostridium Welchii* group.

The thermal death point of spores shows two distinct groups; one whose thermal death point is 87° to 90°, and the other above 100°. Some strains from market milk survive 100° for thirty and forty minutes, while spores from human feces do not survive 100° for fifteen minutes.

There is no correlation between spores and vegetative forms in corresponding cultures, nor is there any relationship between the fermentative reactions and the thermal death points.

Artificial cultivation and cultivation for long periods of time render the organism more resistant to heat. The thermal death points of cultures grown in sterile milk are different from those grown in glucose-liver broth. Sub-cultures in all cases give uniform results under identical conditions.

Cultures from fecal sources are more resistant to heat than those from milk. Pasteurization kills all vegetative forms of *Clostridium Welchii*.

XI. PATHOGENICITY

1. Historical. Since the spores of *Clostridium Welchii* have such a wide distribution in nature, in the air, soil and dust, on the bodies of human beings and animals, on food-stuffs and in the intestinal tract and feces of animals, the question of the possible pathological significance of these spores is an important one. A review of the literature since Welch's discovery of the gas bacillus shows not only a wide diversity of opinion in regard to the pathogenicity of this organism but also varied conceptions regarding its toxicity.

In preantiseptic times, especially in military surgery, wound infection by this organism was much more common than today yet cases are being reported at the present time. In early days the infection was thought by some writers to be due to the penetration of air into the tissues but by most investigators to the decomposition of adipose and bone marrow tissues brought about by contact with the atmosphere. Since the observations of Welch and his associates in the '90's, the investigations have demonstrated that the most common and important cause of gaseous phlegmons or emphysematous gangrene is the "gas bacillus" or *Clostridium Welchii*. Fraenkel, 1902, attributed the general symptoms in this infection to an intoxication due to the decomposition products of the infected tissues while Metchnikoff, 1908, Korentchewsky, 1909, Kamen, 1904, Herter, 1907, Passini, 1905 and others ascribe them to ordinary endotoxin absorption. Other views expressed ascribed the locally destructive effects of the gas bacillus to mechanical action or to the production of fatty acids causing acidosis which brought about fatal effects.

Weinberg, who discovered a strain of gas-forming bacillus isolated from cases of gas gangrene among French soldiers in 1916, held the view that the organisms did not cause the gangrene, but that the condition preceded the infection and he has obtained toxic and antioxic products for various anaerobic bacteria isolated from gangrenous wounds. He has also prepared an antibacterial serum with one of the *Clostridium Welchii* group, but failed to detect either the exotoxin or its corresponding antiserum. Kenneth Taylor in 1916 considered that gaseous gangrene is the result of the mechanical action of the gas in a local focus of developing saprophytic bacteria. His conception is that *Clostridium Welchii* attacks the carbohydrates of the muscular tissue and produces a large volume of gas, which, being unable to escape from the tissues, exerts pressure upon the blood vessels, impeding the circulation so that necrosis results. The necrotic tissue is invaded by putrefactive bacteria which disorganize it.

Wright concluded as a result of his researches that *Clostridium Welchii* operates through the production of an acid condition of the blood and tissues through which the antitrypsin is diminished which permits a tryptic digestion of the proteins so that the bacilli are provided with a highly favorable medium of growth. The intoxication which follows is an acidemia according to his views.

Conradi and Bieling have recently distinguished two phases of action of the bacilli, namely, the fermentative and the saprophytic stages. In the fermentative phase, the carbohydrates are attacked, forming lactic, butyric, proprionic and succinic acids which are the immediate causes of the edema and necrosis of the tissues. In the other phase, the spore-bearing organisms give rise to putrefaction of the dead tissues and consequent intoxication.

Stewart West, following a study of an anaerobic bacillus isolated from an infection in a French soldier, concluded that the bacillus produced no true exotoxin nor was the bacterial protein toxic and believed that acid formation in animal tissue was a powerful factor in its injury.

Simonds was unable to produce toxin in artificial media. Cultures from a normal stool were studied and proved very pathogenic for guinea-pigs but no evidence of toxin production was shown. He believed that the products of the bacteria, and those of other bacteria present, damaged or killed more tissue and thus spread the infection. Living tissue was not damaged, but in order that growth might occur a sufficiently anaerobic condition must be supplied by the dead tissue.

Westenhoffer claims that *Clostridium Welchii* is a pure saprophyte and produces its effects in dead tissue only. For its occurrence unfavorable conditions in the tissues are essential.

Researches by Bull and Pritchett in April and November 1917 have completely overthrown all the conceptions previously held of the manner of the pathogenic action of this organism. Experiments recently performed by them seem to render all the above theories untenable and to establish the fact that the local destruction of tissues and the lethal effects of *Clostridium Welchii* are due to a specific bacterial toxin and not to a blood invasion of the micro-organisms nor to acid intoxication. The work shows definitely that under suitable cultural conditions (broth plus sterile non-denatured muscle and + 0.1 per cent glucose incubated less than twenty-four hours) *Clostridium Welchii* produces a soluble toxin which after separation from the bacilli, is capable of causing the characteristic lesions and possesses the physical and biological properties of an exotoxin. Furthermore, infections can be successfully prevented and controlled by neutralizing the toxin with a specific immune serum as proved by these writers.

2. Experimental. The present investigation on pathogenicity is an attempt to differentiate between the human and bovine types and also to present evidence which will substantiate the claims of Bull and Pritchett that the local infection and lethal effects of *Clostridium Welchii* are due to a specific bacterial toxin which can be neutralized with a specific antitoxin.

The modes of inoculation by which the pathogenic properties were demonstrated for *Clostridium Welchii* were subcutaneous, intraperitoneal and intravenous injections of fresh cultures. The

injection of 2 cc. of a virulent glucose broth culture, grown at 37.5° for eight to ten hours, into the subcutaneous tissue of the abdomen of a guinea-pig gave rise to typical gas gangrene and caused the death of the pig in from twelve to thirty hours, depending on the virulence of the culture. When pure cultures of virulent organisms are injected subcutaneously the injection leads to necrosis of all the tissues surrounding the point of inoculation with the presence of rapidly spreading gas phlegmons, giving rise to a local swelling and liquefying necrosis of the involved muscle and skin. The hair of the pig around the inoculated area pulls out very easily, exposing the skin which is free from the body wall. If the animal survives, a large abscess is formed which later bursts, and recovery occurs with eventual sloughing and cicatrization. At autopsy after death an abundant sero-sanguinous exudate is formed extending along the entire abdominal cavity containing *Clostridium Welchii* in pure culture with very few pus cells. There is an extensive muscle necrosis and disintegration of the fat extending from the injection along the greater part of the abdomen and in some cases extending along the legs and up to the neck. The abscess may extend along the entire abdominal and thoracic regions. Muscles and connective tissues are so badly necrosed that they assume a semi-liquid condition filled with gas bubbles, giving off a characteristic strong butyric acid odor. The amount of gas varies very much, in some cases there may be only a few bubbles, and in other cases tissues everywhere are blown up with gas. After death there may be a rapid swelling of the subcutaneous tissues and gas bubbles may be found in the heart, blood vessels and other organs. Certain organs, especially the liver, offer more favorable food supply for growth and development with gas formation than do others, but even in the liver, cases occur where bacilli are present without gas. In the spleen and kidneys gas bacilli are present in clumps, at times, surrounded by necrotic areas with gas formation. The bacilli are most numerous in the disorganized and necrotic tissue, fewer in the bloody exudate and very few or absent in the heart's blood. Cultures were also recovered from the intestines and sometimes from the stomach

and pancreas. Smears showed the presence of capsules and Gram-positive bacilli from the serous fluid and disorganized tissues upon microscopical examination. The gas bacillus seems to select certain tissues and organs of the body, namely, loose connective tissue, voluntary muscles and liver, perhaps on account of the glycogen which they contain. Gas may appear in the tissues as early as eight to ten hours after inoculation.

A larger amount of culture is necessary for infection when given intraperitoneally than when given subcutaneously or intravenously yet if lethal doses are used, the same effect takes place in the tissues and body fluids. The lethal dose in intraperitoneal injections was 0.25 cc. for a 607-gram guinea-pig or 0.04 cc. for a 100-gram pig. The minimum lethal dose for subcutaneous and intravenous injections was 0.1 cc. for 400 to 450-gram guinea-pigs. 0.1 cc. was the smallest amount that could be inoculated with the needles available.

The laboratory animal employed for the most part to test the pathogenicity of *Clostridium Welchii* was the guinea-pig, the guinea-pig being more susceptible to infection that the rabbit. As far as possible pigs weighing about 500 grams were used which had not been previously used for experimentation. The subcutaneous injection was adopted chiefly because it proved more successful than the intraperitoneal. The rabbit is more resistant to local infection than is the guinea-pig but nevertheless lesions can be produced by virulent cultures provided larger doses are given.

Subcutaneous inoculation of virulent cultures of *Clostridium Welchii* when injected into a guinea-pig will produce severe local infections, terminating fatally in from ten to thirty-six hours in the majority of cases, the more virulent the organism the quicker being the reaction. In exceptional cases, death has occurred after five to nine days with the recovery of the organism from the tissues at autopsy. In one case a 353-gram pig received 1 cc. injection of number three milk culture and lived for eleven days, having lost 140 grams and developing an abscess over the entire abdominal region which gave off a strong, offensive, putrefactive odor. In cases of sublethal doses or non-pathogenic cultures a

mild local infection occurs from which recovery takes place in a very few hours or the pig remains inactive with no appetite for several days. The injection causes local swelling which after two to three days forms a large abscess and breaks, exposing the abdominal wall. After the abscess opens the pig gains steadily, recovering completely in the course of five to seven days, while the abscess heals and leaves a clean scar.

Cultures of *Clostridium Welchii* vary greatly in virulence. All strains showing any virulence are pathogenic for guinea-pigs, while some may not cause fatal infection in rabbits. The virulence of a strain is increased by passage through the animal body. The source from which an organism was isolated gives no absolute clue as to its pathogenicity. However, of the 9 strains of *Clostridium Welchii* isolated from the feces of man all have proven very highly pathogenic for guinea-pigs and in relatively larger doses, are pathogenic for rabbits. Of the 12 from cow feces, 8 are non-pathogenic and 4 are highly pathogenic. These 4 strains were isolated from 4 cows in the same herd.

The milk cultures used in the test for pathogenicity showed a difference in virulence. Of the 12 cultures tested, 8 were pathogenic in varying degrees; 2 in twelve hours, 2 in forty-eight hours, 1 in eighty hours, 1 in five days, 1 in nine days and 1 in eleven days. Four cultures from milk were grown for twenty-four hours in sterile milk instead of glucose broth before the injection of the animal to see if different culture media would effect the pathogenic result. Apparently growth in sterile milk gives the same result as in glucose broth for the strain was non-pathogenic under both conditions.

Controls were run to check up the results in all cases. The pathogenicity of spores of *Clostridium Welchii* was tested in five cases and gave negative results. To assure the inoculation of spores alone, the cultures containing spores were heated to 80° for fifteen minutes in order to kill all the vegetative forms before the injection into the animal. The spore cultures tested were taken from two strains of *Clostridium Welchii*, one from human feces and the other from market milk, both being virulent for guinea-pigs in the vegetative form. Four of the experiments

were performed on guinea-pigs by injecting the heated spores subcutaneously in 1 and 5 cc. quantities. In each case the animals increased in weight and showed no apparent reaction. The fifth experiment was an intravenous injection of 10 cc. of a spore culture from human feces in a rabbit weighing 2200 grams with negative results.

From these results it is safe to assume that spores heated to 80° for fifteen minutes do not readily germinate in the circulation or in living tissue. Apparently when spores of *Clostridium Welchii* gain entrance to living tissue, they adapt themselves to the amount of oxygen present to such a degree that they will survive for a time but do not produce infection. Thus the healthy human body is protected against infection by the spores of *Clostridium Welchii* which are so widely distributed. In the case of injury to the tissues such as wounds, fractures, etc., there is a sufficient anaerobic condition in the dead tissue to allow the organism carried into the wound to begin to grow and produce its characteristic lesions.

Previous to the investigations of Bull and Pritchett, 1917, no explanation could be given for the fact that spores would not germinate and produce fatal results. When vegetative forms were injected in sufficient quantities in living tissue, inflammation and necrosis of the subcutaneous tissues and muscles developed causing death but no reason could be found for the behavior of spores. Bull and Pritchett, 1917, determined the fact that the toxicity of the fluid is destroyed by heating for 30 minutes at 70° and is greatly diminished at 62° for a similar period. Therefore, it is probable that the toxin was destroyed by heat in these experiments on the effect of the inoculation of spores and hence no necrosis or local lesions were produced. This fact strengthens the view that a toxin is produced during the growth of the bacillus.

3. Effect of acid upon the tissues. The following experiments were conducted to determine whether or not the presence of acid in the culture has any effect upon pathogenicity. Cultures grown for eight to ten hours were injected in most cases, while in a few cases twenty-four hour cultures were used. The acidity

was not calculated in every case before inoculation but those tested showed acidities (Fuller's scale) ranging from 1.2 to 3.5 per cent in terms of N NaOH with phenolphthalein as an indicator. In the case of the twenty-four hour culture where the greatest acidity was produced, mild local infections occurred and in one case death after nine days. In one instance the amount of acid produced by the multiplication of the bacilli in the tissues was determined. Death occurred in twenty hours after the injection of a lethal dose and at autopsy the tissue surrounding the necrotic area was titrated with phenolphthalein as an indicator. It was found that the acidity had reached approximately 12 per cent before the death of the animal, showing again the ability of *Clostridium Welchii* to produce acid by means of utilizing the carbohydrates in the tissues. In two cases, the virulent broth cultures were neutralized with NaOH which when injected in lethal doses in the subcutaneous tissues of guinea-pigs proved fatal, presenting the same symptoms and effects as the unneutralized cultures. The same effects were produced if the sedimented bacilli were injected or the supernatant fluid, provided a sufficient dose was given. Although sufficient experiments have not been performed for definite conclusions regarding the rôle of acidity, yet the results already at hand point conclusively to the exclusion of this factor in bringing about the fatal effects. If the acidity factor aids in the destruction of the tissues, the effect is secondary in the production of fatal results.

4. Discussion of results. Animals are more susceptible, and smaller doses are more liable to kill, when the inoculations are subcutaneous or intramuscular than when other modes of inoculation are employed. The difficulty encountered with the intravenous inoculation for guinea-pigs made it impracticable for routine work. In the one successful attempt, however, it proved very fatal, 0.1 cc. being sufficient to kill a 500-gram guinea-pig. The minimum lethal doses for virulent cultures when injected subcutaneously and intraperitoneally are 0.1 cc. and 0.25 cc. respectively for 600-gram guinea-pigs. The minimum lethal dose was not determined for rabbits.

The guinea-pig is peculiarly susceptible to infection while the rabbit is much more resistant. The rabbit succumbs, however, to the inoculation of a more virulent strain or a larger dose.

The duration of the disease in these animals varies from a few hours to several days, complete recovery from a local infection taking one to three weeks. All degrees are observed from a very mild local infection to a severe necrosis, resulting in the liquefaction of the muscle and tissues involved, accompanied by abundant gas production, which results in prostration, complete collapse, and death in a short time. The degree of infection depends on the virulence of the culture.

While no experiments were made to determine the presence of a soluble toxin, yet by eliminating certain factors evidence points strongly to the fact that the local infection and lethal effects of this organism are due to a specific bacterial toxin similar to that of the diphtheria bacillus, as already demonstrated by Bull and Pritchett, 1917. The factor of acidity of the culture appears to be secondary in its effect to its toxic action.

Table 9 gives the results of 55 experiments made on the pathogenicity of cultures of *Clostridium Welchii* isolated from the following sources, human feces 9, cow feces 10 and milk 11. Two cubic centimeters of an eight-hour glucose broth culture were inoculated subcutaneously.

SOURCE	TOTAL TESTED	PATHOGENIC	NON-PATHOGENIC	PER CENT PATHOGENIC
Human	9	9	0	100.0
Cow	10	4	6	40.0
Milk	11	8	3	72.7

Spores from cultures obtained from market milk and human feces, after heating to 80°–82° for fifteen minutes to kill the vegetative forms, did not germinate when injected into the circulation or subcutaneous tissues of guinea-pigs or rabbits and the animals showed no reaction.

TABLE 9

Pathogenicity experiments

WEIGHT	SEX	AMOUNT	STRAIN	SOURCE	ACIDITY	REMARKS
				Subcutaneous inoculations		
grams		*cc.*			*per cent*	
186	♀	1.25	6	Human feces	2.5	Fatal in 24 hours. Weight, 186 grams
204	♂	0.75	6	Human feces	2.5	Fatal in 30 hours. Weight, 191 grams
263	♀	0.5	6	Human feces	2.5	Fatal in 12 hours. Weight, 252 grams
367	♂	0.1	6	Human feces	2.5	Fatal in 26 hours. Weight, 317 grams
491½	♂	2.0	4	Human feces	2.3	Fatal in 10 hours. Weight, 468.5 grams
518½	♂	2.0	5	Human feces	2.4	Fatal in 15 hours. Weight, 481 grams
548	♂	2.75	7	Human feces	2.0	Fatal in 15 hours. Weight, 512 grams
550	♂	2.0	13	Cow feces	2.3	Recovered. Reaction slight. Loss in weight
676	♂	0.5	12	Cow feces	1.8	Recovered. Reaction slight. Loss in weight
443	♂	2.0	11	Cow feces	1.2	Recovered. Reaction slight. Loss in weight
443	♂	2.0	8	Cow feces	2.0	Recovered. Reaction slight. Loss in weight
571½	♂	2.0	7	Cow feces	2.0	Recovered. Reaction slight. Less in weight
741	♂	3.0	5	Cow feces	2.0	Recovered. Reaction slight. Loss in weight
499	♂	2.0	5	Cow feces in st. milk		Recovered. Reaction slight. Loss in weight
425	♂	2.0	64	Cow feces		Fatal in 12 hours. Weight, 423 grams
476	♂	2.0	72	Cow feces		Fatal in 27 hours. Weight, 427 grams
548	♂	2.0	65	Cow feces	2.3	Fatal in 12 hours. Weight, 543 grams
616	♂	2.0	71	Cow feces		Fatal in 13 hours. Weight, 590 grams
516	♂	2.0	E 11	Human feces	2.0	Fatal in 13 hours. Weight, 489 grams
540½	♂	2.0	E 8	Human feces	2.1	Fatal in 25 hours. Weight, 502 grams
523	♂	2.0	G 8	Human feces	2.1	Fatal in 38 hours. Weight, 478 grams

510½	♂	2.0	N 6	Human feces		Fatal in 39 hours. Weight, 469 grams
773	♂	2.0	G 8	Human feces		Fatal in 32 hours. Weight, 770 grams
455	♀	0.5	$22\frac{7}{16}$	Milk	2.5	Fatal in 46 hours. Weight, 439 grams
448½	♀	1.0	$21\frac{1}{15}$	Milk	2.4	Fatal in 80 hours. Weight, 360 grams
656	♂	2.0	$6\frac{7}{16}$	Milk	2.4	Severe reaction. Abscess. Recovery. Lost 125 grams
666	♂	2.0	$11\frac{7}{16}$	Milk	2.0	Severe reaction. Abscess. Recovery. Lost 140 grams
551	♂	2.0	$8\frac{7}{16}$	Milk	2.5	Severe reaction. Abscess. Recovery. Lost 45 grams
566	♂	2.0	$E\frac{7}{16}$	Milk	1.9	Fatal in 5 days. Weight, 482 grams
322	♀	1.0	$1\frac{7}{16}$	Milk	1.8	Fatal in 12 hours. Weight, 311 grams
306	♀	1.0	$18\frac{7}{16}$	Milk		Fatal in 12 hours. Weight, 300 grams
280	♂	0.5	$8\frac{7}{16}$	24 hour culture	3.0	Recovered. Reaction quite mild. Slight loss
300	♀	0.5	$19\frac{7}{16}$	24 hour culture		Fatal in 48 hours. Weight, 285 grams
353	♂	1.0	$3\frac{7}{16}$	24 hour culture	2.5	Fatal in 11 days. Weight, 219 grams
391	♂	1.0	$12\frac{7}{16}$	24 hour culture	2.5	Fatal in 9 days. Weight, 209 grams
				Tests on pathogenicity for rabbits		
2490	♀	5.0	6	Human feces	2.4	Sublethal dose. Mild reaction. Recovery
2300	♀	10.0	6	Human feces	2.4	Sediment pathogenic in 36 hours. 2150 grams
2150	♂	8.0	6	Human feces	2.4	Liquid pathogenic in 28 hours, 1975 grams
				Intraperitoneal inoculations		
293	♀	0.5	6	Human feces	2.5	Fatal in 10 hours. Weight, 279 grams
607	♀	0.25	6	Human feces	2.5	Fatal in 10 hours. Weight, 580 grams
473½	♀	0.1	6	Human feces	2.5	Recovery. Some reaction. Loss in weight
624½	♂	0.1	6	Human feces	2.5	Recovery. Reaction quite severe. Lost 80 grams
505½	♂	1.5	4	Human feces	2.3	Fatal in 10 hours. Weight, 484 grams
596	♂	3.0	5	Human feces	2.4	Fatal in 10 hours. Weight, 572 grams
553	♂	0.2	12	Cow feces	1.8	Recovery. Gangrenous. Abscessed. Lost 60 grams
550	♂	2.0	12	Cow feces	1.8	Recovery. Gangrenous. Abscessed. Lost 40 grams
561½	♂	2.0	13	Cow feces	2.3	Recovery. Gangrenous. Abscessed. Lost 40 grams

TABLE 9—*Concluded*

WEIGHT	SEX	AMOUNT	STRAIN	SOURCE	ACIDITY	REMARKS
				Intravenous inoculations		
grams		*cc.*			*per cent*	
385½	♂	0.5	6	Human feces	2.5	Recovered quickly. Reaction mild. Small abscess
477	♀	0.1	6	Human feces		Recovered after 10 days reaction. Abscessed
449	♀	0.1	6	Human feces		Fatal in 36 hours. Weight, 402 grams
				Subcutaneous inoculations of spores		
2490	♀	5.0	6	Human	Rabbit	No reaction. Gained weight consistently
2200	♂	10.0	6	Human	Rabbit	Intravenous inoculation. No reaction
645½	♂	5.0	6	Human	Guinea-pig	No reaction after initial effect of inoculation
522	♂	1.0	6	Human	Guinea-pig	Very slight loss in weight. No ill effects
442	♀	1.0	$1\frac{7}{16}$	Milk	Guinea-pig	No ill effects. Gained weight
				Effect of acidity on toxicity		
540	♂	2.0	E 8	Human	2.1	Fatal in 25 hours. Subcutaneous. Weight, 502 grams
550	♂	2.0	E 8	Human	Neutral	Fatal in 20 hours. Subcutaneous. Weight, 505 grams
491	♂	2.0	4	Human	2.4	Fatal in 10 hours. Subcutaneous. Weight, 468 grams
518	♂	2.0	4	Human	Neutral	Fatal in 15 hours. Subcutaneous. Weight, 481 grams
				Feeding experiments		
425	♀	2.0	6	Human		Fed spores. Gained with no ill effects
401	♀	1.0	6	Human		Fed vegetative. Gained with no ill effects
449	♀	2.0	6	Human		Fed vegetative. Gained. No spores in feces
270	♂	1.0	E 8	Human		Fed vegetative. Gained. No spores in feces
261	♂	2.0	E 8	Human		Fed vegetative. Gained. No spores in feces

XII. IMMUNITY

In the literature dealing with the immunity of animals to inoculation with *Clostridium Welchii* and the production of immune sera, we find views as varied and unreliable as in regard to the other properties of this organism. Very little work has been done on the production of artificial immunity. Previous to 1916, investigators were unable to protect guinea-pigs against infection by injections of bacilli killed either by heat or by chemical substances. A pig which had recovered from one inoculation was believed to be more susceptible to a new injection.

On the other hand Rosenthal and Theroloix, 1909, immunized horses against *Clostridium Welchii* and produced a serum which protected rabbits against intrapleural and intramuscular injections of the living organisms. These results were not in line with other observations made at that time yet later evidence seems to justify their validity.

Robertson, 1916, undertook experiments to determine whether or not immunity could be produced by the prophylactic inoculation of *Clostridium Welchii* and his results showed no immunity in guinea-pigs, and also indicated that the survival of one infection did not confer any immunity at a later period.

As far as is known Bull and Pritchett, 1917, are the only ones who have shown that the toxic products of *Clostridium Welchii* exhibit antigenic activities and give rise to the formation of active antitoxic substances which possess protective and curative properties. Conclusions from their work show that animals may be immunized actively so as to yield an immune serum which neutralizes the toxin perfectly and in multiple proportion.

As stated, different strains vary greatly in virulence and the same strain varies according to cultural conditions. The conditions under which five experiments were performed to produce immunity were not sufficiently controlled to render conclusive evidence; in particular, too much time elapsed between inoculations. With all the conditions controlled and standardized, it is believed however that a lasting immunity can be produced for guinea-pigs. A description of the five experiments follow:

1. A male guinea-pig, weighing 571.5 grams was inoculated subcutaneously with a 2 cc. glucose broth culture 7 of cow feces. Although there was a loss in weight to 547.5 grams at the end of the first day, no discomfort was observed and the pig was lively and in a healthy condition. In forty-eight hours it had gained slightly, weighing 553 grams and its weight remained quite constant for the next few days. This injection was made on January 31, 1917, and proved non-pathogenic. On February 6, the same pig weighing 548 grams was reinoculated with a pathogenic strain to see if any immunity had been produced. Two and three-quarters cubic centimeters of number seven culture from human feces was used subcutaneously. A reaction occurred but did not prove fatal. The pig lost weight consistently, dropping to 457 grams on February 19, thirteen days after the inoculation. A local infection occurred with swelling of the abdomen and gas in the tissues. A large open abscess was formed along the entire right side of the abdomen. On February 19, the abscess began to heal and the animal gained consistently, recovering completely in twenty days. A control pig was inoculated with number seven human feces and died in fifteen hours. It was evident, therefore, that immunity had been produced to a high degree by the previous injection of a non-pathogenic strain.

On March, 20, 1917, this same pig now weighing 528 grams and in excellent condition was inoculated again subcutaneously with 2.5 cc. of a fresh culture of a vegetative organism from human feces. Local swelling was produced and loss in weight was consistent to 467 grams when the abscess broke and recovery seemed probable. Two days after another abscess appeared extending to the neck which further weakened the resistance of the pig and resulted in another loss of weight from 467 to 350 grams. Although the abscess had practically healed the pig was found dead on the fourteenth day. The cause of the death was not primarily due to the infection of *Clostridium Welchii*, but after the resistance had been lowered to such an extent the pig was unable to survive. A smaller dose should have been injected, although some immunity was no doubt produced. The control pig died thirty hours after the injection of a 2 cc. culture subcutaneously.

II. A male guinea-pig weighing 499 grams, was inoculated subcutaneously on February 6, with 2 cc. of culture 5 from cow feces grown in sterile milk. A normal infection occurred resulting in gas in the tissues and a pronounced swelling. Loss in weight continued for two

weeks dropping to 428 grams when the abscess healed and the animal returned to its normal healthy active condition, gaining 50 grams in the next week. On March 20, forty-two days after the first inoculation, 1.5 cc. of a fresh culture from human feces, which was pathogenic for a control pig in thirty-nine hours and produced a violent reaction, was injected into this pig which had completely recovered from the reaction of the non-pathogenic strain. After twelve days the pig succumbed having lost weight consistently going from 527 to 267 grams. The intestine and other organs were exposed due to the open abscess extending the entire length of the abdomen with pus filling the subcutaneous tissues. Immunity must have been produced to have kept the pig alive for so long a time while undergoing such a severe infection.

III. A guinea-pig weighing 656 grams was inoculated subcutaneously on February 6, 1917, with 2 cc. of milk culture 6 isolated on July 16, 1916. The pig lost weight and showed the typical lesions with the presence of gas bubbles under the skin. After the first week the abscess which had formed on the fourth day had practically healed but there was another one forming near the former. The pig weighed 524 grams and was apparently healthy. At the end of the second week the pig had completely recovered from the infection and was in excellent condition. This strain although it produced a moderate infection did not kill the pig.

On March 20, 1917, another injection was given this pig, forty-two days after the first. Two cubic centimeters of a very virulent strain G.8 from human feces was used and proved fatal in twelve hours. If any protection had been produced by the previous inoculation, it had run out before forty-two days. This strain, however, was extremely virulent and needed a powerful immunizing influence to counteract its toxic effect.

IV. A pig weighing 666 grams was inoculated subcutaneously on February 6, with 2 cc. of milk culture 11. A characteristic lesion developed in a few hours resulting in a large abscess and loss in weight to 529 grams in two weeks. After the abscess broke, recovery followed rapidly with the healing of the abscess. In three weeks the animal was as lively as ever. On March 20, the pig weighed 578 grams and was injected with 3 cc. of the same pathogen as used in III, namely, G.8, which was highly pathogenic in fifteen hours in a 600-gram control pig. No immunity was present here for death occurred in twenty hours after severe convulsions. Again the dose was too great and the interval between injections too long.

V. On February 6, 2 cc. of milk culture 8 was injected into a 551-gram pig subcutaneously. Although this strain did not prove fatal in two cases, local swelling occurred accompanied with gas and the formation of an abscess. The reaction was not so violent as in some cases although the animal lost 43 grams. Recovery took place in three weeks.

A second injection was given on March 23, 1917, the pig weighing 617 grams. Two cubic centimeters of a twenty-four hour old culture 19, which had previously proved pathogenic for guinea-pigs, was inoculated subcutaneously and failed to produce a fatal necrosis. Emphysematous crackling due to the formation of gas bubbles occurred, but the reaction was not as severe as in other cases. In two weeks the pig recovered from the second injection and had returned to its active condition. Immunity was thus produced against a virulent culture in guinea-pigs from the same source, namely, milk.

From these five experiments it is evident that immunity can be produced by the injection of avirulent cultures or sublethal doses of virulent organisms.

XIII. EFFECT OF FEEDING CLOSTRIDIUM WELCHII

Previous investigators have met with little or no success by feeding *Clostridium Welchii* to animals. In a few cases diarrhea has been induced in young guinea-pigs and kittens but attempts to cause ill effects in adult animals have met with negative results. Before determining the effect of feeding cultures to guinea-pigs, feces from several animals were collected and tested for the presence of spores and vegetative forms of *Clostridium Welchii*. The feces from guinea-pigs, weighing approximately 400 grams, were collected in the following manner. The sides and belly of the pig were washed with a 5 per cent carbolic acid solution and then the pigs were placed in the sterile container one at a time, and a sample of feces collected. After each sample was taken, the container was thoroughly sterilized. After a sufficient quantity of feces had been excreted the pig was released and the feces analyzed as follows:

The sample of feces was added to 100 cc. of freshly sterilized water and shaken vigorously until the pellets were thoroughly

broken up. One-half cubic centimeter of this emulsion was inoculated into 10 cc. glucose-liver broth tubes (five for each sample) and heated to 80° for fifteen minutes and incubated. Five other tubes were inoculated and incubated directly at 37°. Negative results for the presence of *Clostridium Welchii* in the normal guinea-pig were obtained.

To determine the effect of feeding spore and vegetative cultures to adult and young pigs, six experiments were performed, and in no case was the effect fatal. The cultures were mixed with milk alone, and bread and milk. The pigs refused to eat milk alone with *Clostridium Welchii*, but when mixed with bread, they ate heartily. In the two experiments where the spore cultures were fed there were no apparent disturbance, the pigs seeming as active and healthy as under their normal diet. The feces were normal and hard, and no diarrhea nor intestinal trouble developed. Samples of the feces were analyzed daily for the presence of spores, and vegetative forms. Spores were recovered for three days, after which negative results were obtained. During this time the animals gained weight and seemed to thrive upon their new diet. Two cubic centimeters and 4 cc. of spores were fed from virulent strains to adult guinea-pigs weighing 425 and 445 grams respectively, with negative results.

With vegetative cultures, four experiments were performed using two adult pigs weighing 401 and 449 grams respectively, and two younger pigs, about three months old weighing 270 and 260 grams respectively. Feces before feeding did not contain *Clostridium Welchii*. One cubic centimeter and 2 cc. of a highly virulent strain for guinea-pigs were used in the feeding, soaking bread with milk and adding the culture of *Clostridium Welchii* grown in glucose-liver broth for eight hours. The difference in the age of the pigs did not seem to change the effect on the feeding, for in the fourth pig no discomfort was observed, and a steady gain in weight was recorded. Analysis on the first day showed numerous vegetative forms and the absence of spores in the intestine. The second day after feeding, *Clostridium Welchii* was recovered in all cases as spore. Negative results were again obtained in five days.

The very interesting conclusion from these experiments is that vegetative forms sporulate in the intestines when present either as a result of artificial feeding or inoculation. In all cases of subcutaneous inoculation of vegetative cultures, spores were observed in different parts of the intestine. From the six feeding experiments no ill effects were observed and cultures disappeared quickly from the intestines.

XIV. CONCLUSIONS

1. *Clostridium Welchii* is a non-motile, straight rod, 3 to 6 micra in length and to one and one-half micra in breadth, with the ends slightly rounded or square cut. It stains readily with the ordinary dyes and is Gram positive. Capsules are present in cultures made directly from body fluids or body tissues, from milk or feces, but disappear in succeeding cultures.

2. Sporulation of *Clostridium Welchii* takes place in a great variety of media, provided no fermentable substance is present. Sporulation never occurs in the tissues, organs and body fluids of the living animal but does take place in the intestine. The spores are usually oval, from 1.5 to 3 micra long and form in the middle of the rod or slightly toward one end. The spores germinate when grown in media containing glucose, galactose, lactose, sucrose, maltose, dextrin and starch. They withstand a wide range of reaction, at least from -2 to $+12$. They will germinate in slightly alkaline or acid media but best in neutral media.

3. *Clostridium Welchii* is an obligate anaerobe which requires strict anaerobiosis for its growth. Boiling the media just before using renders it suitable for the growth and isolation of *Clostridium Welchii*. For continued growth on solid media, the surface should be covered with sterile paraffin or other methods for preserving anaerobic conditions should be employed.

4. *Clostridium Welchii* is capable of growth upon all of the ordinary culture media but grows better in the presence of 1 per cent glucose or maltose. Vegetative forms must be transplanted every forty-eight hours. In sugar-free media sporulation occurs in two or three days. The organism does not grow in

peptone-free media. Spores of *Clostridium Welchii* can be preserved indefinitely in all media in the absence of a fermentable substance.

5. *Clostridium Welchii* develops best at 37° to 38°C. Growth does not occur above 42°C. nor below 10°C. From 18° to 35°C. growth is abundant but slow. Freezing vegetative and spore forms effects a steady reduction in numbers which leads to complete destruction of vegetative forms in seven or eight days and of spores in ten to twelve days.

6. Surface colonies of *Clostridium Welchii* in plain and sugar agar are opaque white, grayish white or brownish white in color, by transmitted light, sometimes with a central darker dot. Deep colonies are spheres or ovals. Colonies vary in size from 0.5 to 2 or 3 micra in diameter.

7. Gelatin and blood serum are liquefied. Gas and acid are produced from glucose, galactose, lactose, maltose, sucrose, dextrin and starch. Milk is coagulated with a characteristic "stormy fermentation" in twenty-four to forty-eight hours at 37°. The curd is later digested. *Clostridium Welchii* is chiefly a fermentative organism and attacks proteins only in the absence of a fermentable carbohydrate.

8. The thermal death point of vegetative forms of *Clostridium Welchii* varies from 56° to 63° for a fifteen minute period of exposure. The thermal death point of spores shows two distinct groups; one whose thermal death point is from 87° to 90° and the other which survives 100°.

9. The minimum lethal dose in intraperitoneal injections of vegetative forms is 0.04 cc. of an eight-hour broth culture for a 100-gram guinea-pig, while for subcutaneous and intravenous injections the minimum lethal dose is 0.025 cc. The rabbit is more resistant to infection than is the guinea-pig. Cultures of *Clostridium Welchii* vary greatly in virulence. Cultures containing spores heated to 80° for fifteen minutes are non-pathogenic. Immunity can be produced by the injection of avirulent cultures or sublethal doses of virulent organisms. Feeding vegetative or spore forms has no ill effect.

10. On the basis of acid production and spore formation in glycerol and inulin broths members of the *Clostridium Welchii* group may be divided into four sub-groups.

11. *Clostridium Welchii* is a normal inhabitant of the intestines and is very widely distributed in nature in the spore form. Milk direct from the cow is free from *Clostridium Welchii* but becomes contaminated through uncleanly methods and carelessness in handling so that market milk at the time of delivery consistently contains spores of this organism. The examination of 525 samples of milk indicate that *Clostridium Welchii* is present in most market milk.

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A NOTE ON THE VIABILITY OF MENINGOCOCCI ON YEAST AGAR MEDIUM

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In 1918, the writer showed that a simple yeast medium (extract of baker's or brewer's yeast) could be used for prolonging the viability of the meningococcus. The medium possesses the advantages of requiring no meat infusion or meat extract or peptone. It is prepared with agar as a base and is to be used in the solid or semi-solid form. Observations at the time were extended over a period of six, and in several instances over a period of eleven weeks. More than twenty strains of meningococcus, recent and old, representing normal, irregular and para types isolated from the spinal fluid and the nasal mucosa, were tested on the solid medium and were found fully viable after six weeks on the average and for some strains which were observed beyond that time, for eleven weeks, when stored at 37°C. At room temperature, observations were made for one month only and at the end of this time, cultures were still viable. It was believed that a semi-solid medium was best adapted for unusually prolonged viability of organisms such as the meningococcus and other species.

Some recent observations which I wish to report, point to yeast media as a valuable adjunct in routine bacteriological work, particularly in view of the fact that others, notably Ayers and Rupp recently, have confirmed the adjuvant properties of yeast extracts in culture media.

Representatives of the normal, irregular and para types of meningococcus were seeded in semi-solid yeast agar prepared as described in earlier articles by the writer, and stored at 37°C. and at room temperature (24° to 26°) after a preliminary incu-

bation over night at 37°C. Transplants were made to glucose agar at monthly intervals. Luxuriant growth was obtained with all of the strains studied for a period of five months, after which the tests were curtailed. It is preferable, perhaps, to store cultures at incubator temperature in order to maintain a "body environment," but insofar as viability is concerned, there seems to be no difference between the two methods, for the time observed.

No tests have been made to determine the effect of such storage on virulence. A few experiments, however, have been designed with a view to disclosing any possible changes in agglutinogen or agglutinin content of meningococcus. The results indicate that monovalent sera obtained with strains grown on yeast medium for several months do not differ in agglutinating properties from sera developed with cultures which have been grown on the usual media. Similarly, cultures which have been growing on yeast media for a long time are agglutinated by polyvalent sera to the same extent as are cultures grown on other media.

SUMMARY AND CONCLUSIONS.

Meningococcus cultures in semi-solid yeast agar when stored either at room temperature or in the incubator at 37°C., were still fully viable after five months.

In all likelihood the antigenic nature of the organisms remains unaltered as may be indicated by comparative agglutination tests.

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THE USE OF AGAR SLANTS IN DETECTING FERMENTATION

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In a recent paper (Conn, 1919) one of the writers described the use of agar slants instead of liquid media for detecting acid production by two soil organisms, stating the method to be much more satisfactory for these particular bacteria than the old method. Subsequently this method was given as an alternate procedure in the report of the Committee on the Descriptive Chart (1919). We have recently used the method for a great variety of soil and milk organisms with such great success that it seems worthy of more specific mention. The technic is as follows:

One per cent of the desired sugar is added to an agar medium favorable to the growth of the organisms to be tested. The reaction is adjusted by the use of brom thymol blue to a pH-value between 6.8 and 7.2. Then an indicator is added whose range lies just to the acid side of pH = 7.0, such as china blue or brom cresol purple; or better yet, a mixture of the former with cresol red or sodium rosolate (as recommended by Bronfenbrenner, Schlesinger and Soletsky, 1920), or of the latter with cresol red, in order to detect decreases as well as increases in hydrogen-ion concentration. The following concentrations of indicators are recommended: Brom cresol purple 0.001 per cent; Brom cresol purple purple and cresol red each 0.0005 per cent; China blue 0.0025 per cent; China blue 0.0025 per cent plus sodium salt of rosolic acid 0.005 per cent.

It is convenient to keep the indicators in concentrated alcoholic stock solutions of such a strength that a definite amount can be measured out per litre of medium: i.e., a 1.6 per cent stock

solution of brom cresol purple can be made up and used alone at the rate of 1 cc. to the liter of medium or when combined with cresol red at the rate of 0.5 cc. to the liter.

Inoculate both on the surface and in a stab at the base of the slant. The tubes should be watched day by day until the characteristic changes are complete. Changes in reaction can readily be detected by the color; and gas production can be observed by means of bubbles or cracks in the medium if the agar in the tube is sufficiently deep.

With the organisms studied by the writers, this method has had the advantage of showing acid production after a very brief incubation, sometimes in a few hours only. The acid tends to be localized in one spot at first and can be recognized even though it could not be detected if distributed through the entire tube of medium; hence the method is very delicate. Some organisms are characterized by acid production first at the top of the tube; others show the effect first at the bottom. Sometimes the zone of acid is formed at the top and travels slowly to the bottom, followed by a zone of neutral or alkaline reaction. Such changes are generally constant with a given culture.

One of the greatest sources of error in the ordinary test for acid production is the simultaneous production of acid from the sugar and of alkalinity from the peptone. When both of these activities occur in a tube of agar, however, the acid production generally predominates over the production of alkalinity during the early stages of growth, or if not, the two activities are likely to be localized in separate zones of the agar. For this reason, the agar slant method is a great help in overcoming this source of error.

Bronfenbrenner and Schlesinger (1918) recently recommended a method of using indicator agar for detecting acid production, by placing a series of drops in a single petri dish. Their method has the advantage of reducing the amount of glassware used, but it does not show gas production nor the characteristic localization of acid and alkali brought out by the agar slant.

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